COMPARISON BETWEEN PLATE READER AND FLOW CYTOMETRY IN MUTANT ISOLATION FROM CYANOBACTERIA CULTURE

Bioresearch Communications
Volume 12, Issue 1, January 2026

DOI:
doi.org/10.3329/brc.v12i1.86777

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ABSTRACT

Cyanobacteria is promising source of third generation biofules which led to the current interest of genetically modified strains. The isolation of fully segregated mutant cells is slow and tedious due to the presence of multiple chromosome copies Mutant isolation from liquid media can readily accelerate the selection process, although remain as mixed population with varying segregation status. This study was focused on two exploratory approaches of mutant screening from liquid culture via plate reader and FACS. As both the plate reader and FACS can detect cellular fluorescence intensity expressed by a fluorescent reporter protein, four novel fluorescent antibiotic resistance cassettes were constructed via N-terminal tandem fusion with eYFP. The bifunctionality of the fusion proteins was demonstrated by the minimal impact on the extent of antibiotic resistance conferred by the fusions and fluorescence was demonstrated for 3 of the 4 fusions generated. It required only 11 days to isolate eYFP +ve cells from a mixed population (starting at 0.001% eYFP). The optical setting available with the flow cytometer was not optimal to set a gating parameter to detect all the eYFP +ve cells, suggests the necessity of an alternative laser or to change the fluorescent protein. Additionally, two suicide vectors were constructed to incorporate the fusion cassette into cyanobacteria genome by replacing two native sites. The molecular tools and strategies developed in this study can also be applicable in the engineering of other polyploidy bacterial species and thus could benefit the wider scientific community.

KEYWORDS: shuttle vector, fusion protein, genetic transformation, fluorescence, polyploidy

RECEIVED: 16 October 2025, ACCEPTED: 09 November 2025

TYPE: Original Article

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Abbreviations

APC: Allophycocyanin; BASIC: Biopart Assembly Standard for Idempotent Cloning; CDS: Coding sequence concentration; FITC: Fluorescein isothiocyanate; FSC; Forward scatter; IPTG: Isopropyl β-D-1-thiogalactopyranoside**;** RFU: Relative Fluorescence Units; SSC: Side scatter

Introduction

Cyanobacteria is a model organism in synthetic biology to study biological light harvesting, photosynthesis and circadian gene regulation (Hammar *et al.*, 2015). Because of their simple and inexpensive nutrient requirements (mainly water, sunlight and CO_2) (Ruffing, 2011) cyanobacteria are extensively used in numerous industrial prospects like– production of high-value pharmaceuticals, plastics and food or feed additives (Snow and Smith, 2012).

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is one of the most extensively studied species and is considered an ideal host for genetic engineering efforts (Kaneko *et al.*, 1996).

However, following chromosomal integration, obtaining a fully segregated mutant line often requires much longer time than to be expected, as most of the cyanobacterial strains harbor multiple identical copies of chromosome per cell (up to 20 copies in *Synechocystis* sp.) (Schneider *et al.*, 2007). Moreover, chromosome replication in cyanobacteria is asynchronous and little is known about their organization, replication, and segregation.

Based on *Synechocystis* transformation frequency of 10^{-5} (Kufryk *et al.*, 2002), the liquid culture of *Synechocystis* would contain 0.001% transformed cells after a successful transformation. In this case, there will be two major challenges in mutant isolation from liquid media, these are- (1) to identify that 0.001% mutant cell from the liquid heterogenic culture and (2) to ensure the complete segregation of the mutant chromosome before isolation.

In order to isolate the target mutant cells from the potentially heterogenotypic liquid culture, this study focused on two different approaches, include (1) serial dilution techniques with microplate monitoring (via plate reader) of growth and segregation, and (2) fluorescence activated cell sorting (FACS). As both the screening approaches depend on the detection of cellular fluorescence signal, we proposed to modify cyanobacteria with a marker gene that is a fusion of a fluorescent reporter protein with an antibiotic resistance marker. We hypothesized that tandem fusion of a fluorescent reporter protein with an antibiotic resistance marker should result in a cellular fluorescence intensity that correlates with the number of copies (and expression level) of the antibiotic resistance cassette integrated into the cyanobacterial genome, thereby providing a simple fluorescent read-out of the segregation status of a cell. Fluorescence intensity could therefore be used as a marker in plate reader and FACS to select cells with the highest chance of having reached the most advanced segregation state in a heterogenic liquid culture. The fluorescence reporter protein will also assist in visual identification of transgenic cells, whereas, the antibiotic resistant marker will facilitate the selection and maintenance of targeted mutants throughout the study. The proposed high-throughput mutant screening approaches are new in the field of cyanobacterial research, therefore, requires a convenient platform to initiate the final application. The overall aims of this project were to develop strategy and tools required for rapid creation of cyanobacterial mutant based on liquid culture and isolation of mutant cells by selective cultivation. This project was focused on constructing a library of fusion proteins via tandem fusion of a fluorescent reporter protein- eYFP (enhanced yellow fluorescent protein) with an antibiotic resistance protein. The eYFP has a flexible C-terminal stretch of approximately 10 amino acids, however, the N-terminal region is much less tolerant to fusions (Gustafsson *et al.*, 2013). From the crystal structure, it was also observed that the C-terminus of eYFP is quite long and flexible that can assist successful tandem fusion with the N-terminus (the most free end) of antibiotic resistant proteins.

The use of plate reader and FACS technology in order to isolate mutant cells based on the fluorescent fusion protein directed expression has not been reported previously in cyanobacteria research.

Materials and Methods

Genetic materials

All strains and storage vectors used during this study are listed in Appendix I. Oligonucleotide primers are detailed in Appendix II. Plasmid maps are added in the supplementary materials section (Figure S1-4).

Strains and culture conditions

For cloning, rubidium chloride competent *Escherichia coli* DH5α strain (Sigma Aldrich) was used growing at 37°C in Luria Broth (LB) with rotary shaking at 200 rpm, or on LB agar plate supplemented with antibiotics as required: gentamicin (50 µg/ml), ampicillin (100 µg/ml), kanamycin (50 µg/ml), spectinomycin (50 µg/ml) and chloramphenicol (37 µg/ml)

Cyanobacterial strains (collected from University of Dhaka) used in this study are: wild and mutant strains of *Synechocystis* sp. PCC 680. Mutant strain constructed with IPTG promoter (Merck 367-93-1) inducible eYFP (Cambridge Bioscience) and a spectinomycin resistance cassette.

Wild *Syncechocystis* was grown in 30 ml liquid BG-11 media. For mutant strain growth, media was supplemented with spectinomycin (50 µg/ml) and 1 mM IPTG. Cultures were maintained in AlgaeTron (Photon System Incubator) at 30°C with continuous shaking and a light intensity of 50 µE m^{-2} s^{-1}, unless otherwise indicated. *E. coli* cells (50 µl/tube) were transformed by following standard lab protocol.

Molecular biology reagents

Most of the enzymes including- the restriction enzymes (BsaI-HF, DpnI, PstI-HF), *Taq* DNA Ligase, T4 DNA Ligase, Q5 High-Fidelity DNA Polymerase, BSA, dNTPs and NEBuffer 4 were acquired from ThermFisher Scientific and New England BioLab (NEB). Plasmid extraction was carried out using QIAprep® Spin Miniprep kit (QIAGEN) and the gel extraction was performed by using QIAquick Gel Extraction kit (QIAGEN) according to manufacturer's instructions. All the Micro plate based assay assays were carried out in a Tecan Pro plate reader.

Fluorescence assay of Escherichia coli DH5α

In order to evaluate the function of fusion proteins, *E. coli* DH5α cells were grown in 96-well clear flat bottom plates (Costar, 200 µl) at an optical density at 600nm (OD_{600}) of 0.1, at 37°C supplemented with appropriate antibiotics in the range 0 to 100 µg/ml. Analysis was done over Plates were analyzed over a period of 20 hours with a 10 minute temperature equilibrium. Measurements were taken every 5 minutes with a period of orbital shaking (1.5 mm amplitude, 72 seconds) between measurements. Data on fluorescence of eYFP was acquired from 25 flashes per well per time-point by excitation at 503 ± 9nm and emission at 540 ± 20nm using a gain of 75.

Growth assay of wild-type Synechocystis

Cultures of Wild-type *Synechocystis* was grown in 24 well plates (CELLSTAR, 1.5 ml) at 730nm (OD_{730}) of 0.1 in liquid BG-11 medium supplemented with the appropriate antibiotics (0-10 µg/ml). Measurements were taken twice daily until the cultures reached in a stable stationary phase.

Fluorescence assay of cyanobacteria mixed culture

For mutant screening via plate reader, the wild type (Wt) *Synechocystis* was mixed with CB1-RBS5-eYFP strain (Mut) in a way that gave similar number of transformed cells with frequency of 10^{-5} (Viola *et al.*, 2014). Cultures were supplemented with 1 mM IPTG and 50 µg/ml of spectinomycin. It can be expected to have 0.001% recovery of the transformants with 10^{-5} transformation frequency. Based on this idea, cells were mixed in a manner where each of the four rows of 24 well plate contained mixed cultures having 0%, 0.1%, 0.01% and 0.001% Mut, respectively. From unpublished data of the lab, it was assumed that the cultures having an OD_{730} of 1 contain ~$4x10^7$ cells/ml Therefore, with starting OD_{730} of 0.1, the wells containing 0.001% Mut would theoretically give ~ 6 mutant cells out of 6 x 10^7 cells in 1.5 ml culture. The data was acquired by multiplying the fluorescence with the OD_{730}. Measurements were taken twice daily until the cultures reached in a stable stationary phase.

For chlorophyll autofluorescence measurement, the excitation and emission spectra were set at 440nm ± 9nm and 660 ± 20nm, respectively.

Recombinant DNA construction

All the parts of the fusion proteins were ligated by Gibson assembly by following the protocol of Gibson *et al.*, 2009. The

assembly protocol is followed as depicted in the main article with slight modification, described in the result section (Storch *et al.*, 2015).

Flow cytometry data acquisition

The flow cytometry experiments were carried out with *Synechocystis* – Wt and eYFP mutant (both uninduced and induced with IPTG). When required, cells were fixed with 1% formaldehyde (Fisher Scientific) with 10 minutes incubation. Fixed cells were washed twice and resuspended in BG-11 media. Samples were adjusted at OD_{730} = 0.13.

Data acquisition was done using BD Fortessa flow cytometer (Becton Dickinson). The YFP signal was detected using the 488nm laser with a 505 longpass mirror and a 550/30 bandpass filter in front of the detector. All dot plots were recorded using logarithmic scales.

Results

A. Molecular tool development

To generate a library of fusion proteins four different BASIC assembly compatible antibiotic resistance (Ab^R) cassettes were used (mentioned in strains section). All cassettes were standardized and cloned into pJET 2.1 storage plasmid for future use.

To make the cassette BASIC compatible, the GmR cassette was amplified with primer "BASICsuf-b2" and "GmR-mod2" flanking with the *i*P and *i*S region (amplicon size 0.92 kb).

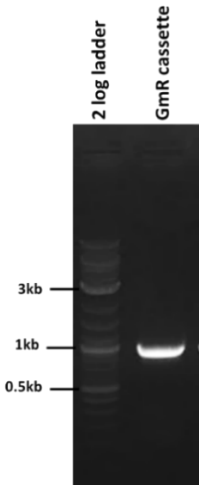

Figure 1. PCR amplified GmR cassette flanking with iP and iS sequence

The amplified fragment gave band near 1 kb (Figure 1). Following gel extraction, the standardized GmR was cloned into pJET 2.1.

Construction of tandem fusion protein library

The tandem fusion proteins contained two physico-chemically compatible fusion partners- eYFP (enhanced Yellow Fluorescent Protein) and an antibiotic resistance (Ab^R) marker. pJET 2.1 storage plasmids containing the respective DNA parts were extracted from *E. coli* in order to ligate the Ab^R backbone and the eYFP CDS with each other. Successful amplification was confirmed by gel electrophoresis where, Ab^R backbones gave bands roughly at 4 kb position and eYFP fragments complementary to a specific backbone gave band at ~800 bp position (Figure 2a, b).

DNA fragments were column purified to perform Gibson assembly aiming to construct the vectors carrying different fusion protein encoding genes. Fusion proteins created in this study are shown in table 1.

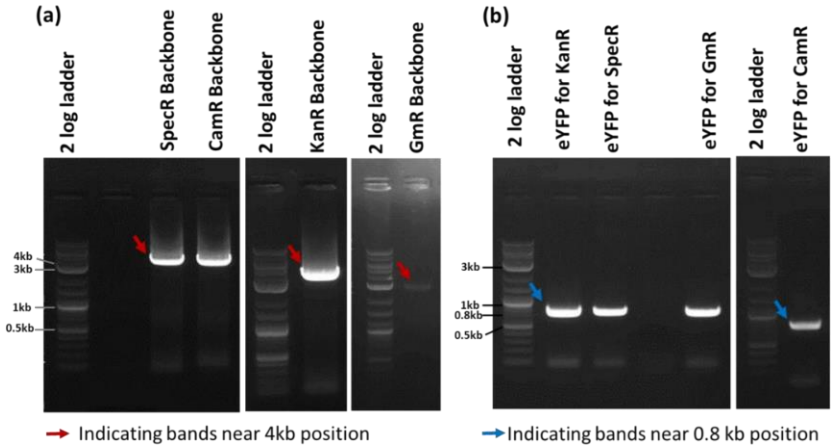

Figure 2. PCR amplified Ab^R backbones and the corresponding eYFP CDS **(a)** Showing the bands of Ab^R backbones (~ 4kb), **(b)** Showing the bands of eYFP CDS (~ 0.8kb).

Table 1. The library of fusion proteins.

	Name	**Abbreviations used later**
1.	Spectinomycin Resistance eYFP	SpecR eYFP
2.	Kanamycin Resistance eYFP	KanR eYFP
3.	Gentamicin Resistance eYFP	GmR eYFP
4.	Chloramphenicol Resistance eYFP	CamR eYFP

For the presence of eYFP marker in the assembly constructs, desired colonies grown on the selective media were identified visually (using a dark reader). The presence of desired fragments in the plasmids were further confirmed by restriction digestion with PstI restriction enzyme. (Table 2, Figure 3). Amplicon size of the backbones are presented in Appendix I.

Table 2. Expected fragment sizes derived after the PstI digestion

Plasmids	**Total plasmid size**	**First fragment size**	**Second fragment size**
KanR eYFP	**(**4704**bp)**	**(**2961**bp)**	**(**1743**bp)**
CamR eYFP	4686	3948	738
SpecR eYFP	4762	4050	712
GmR eYFP	4611	3679	932

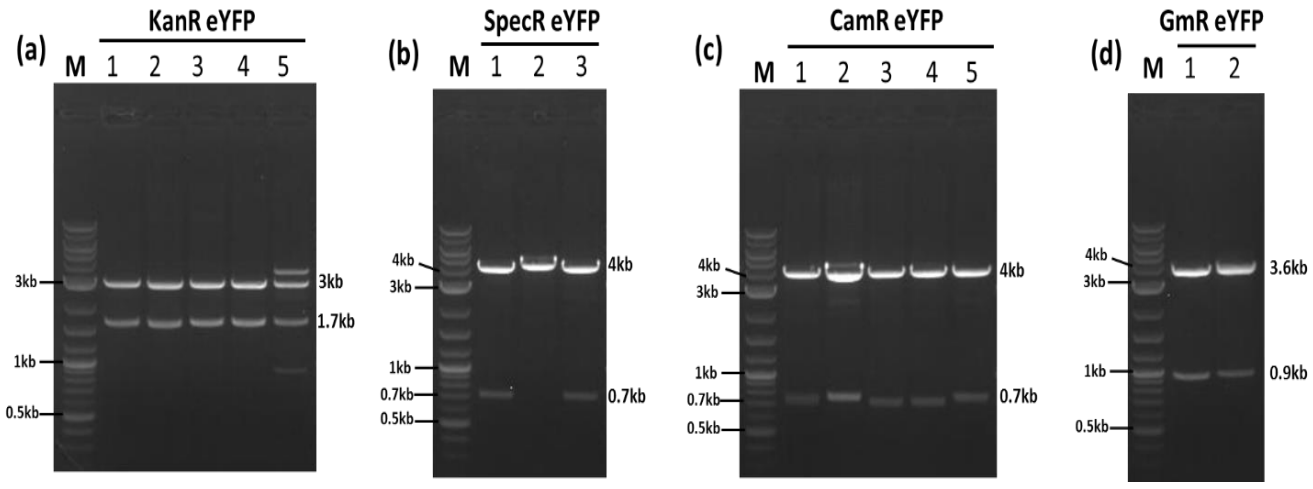

Figure 3. Confirmation of the presence of both Ab^R and eYFP region in the construct by PstI digestion. **(a)** KanR eYFP **(b)** SpecR eYFP **(c)** CamR eYFP and **(d)** GmR eYFP. Theoretical lengths of cleaved fragments are shown on the right side of the gel. M= Marker (2-log ladder).

The existence and orientation of eYFP fragment were further confirmed via sequencing by CloneJET PCR Cloning Kit (Fermentas) (Appendix II).

Functional verification of the fusion proteins

a. Antibiotic susceptibility of transformed *E. coli* DH5α

Growth of transformed *E. coli* DH5α with the original Ab^R cassettes and with the tandem fusion versions was assessed in the presence of increasing antibiotic concentrations.

For KanR, CamR and GmR expressing strains of *E. coli*, there was very little impact on growth at any antibiotic concentration tested (Figure 4a, c, d). The growth rate of SpecR eYFP cells decreased considerably with the increasing antibiotic concentration resulting in a delayed exponential phase compared with cells carrying original SpecR cassette (Figure 4b). The GmR eYFP transformed cells showed almost similar growth pattern to the cells having the original Gm cassette,

although a substantial decline in growth was observed at 100 µg/ml Gm (Figure 4d). However, that the usual working concentration of gentamicin used for resistant strains is 5-20 µg/ml (ATCC, 2004; Poteete *et al.*, 2006). Hence, it is anticipated that the inhibited growth observed in 100 µg/ml concentration will not create any problem in this study.

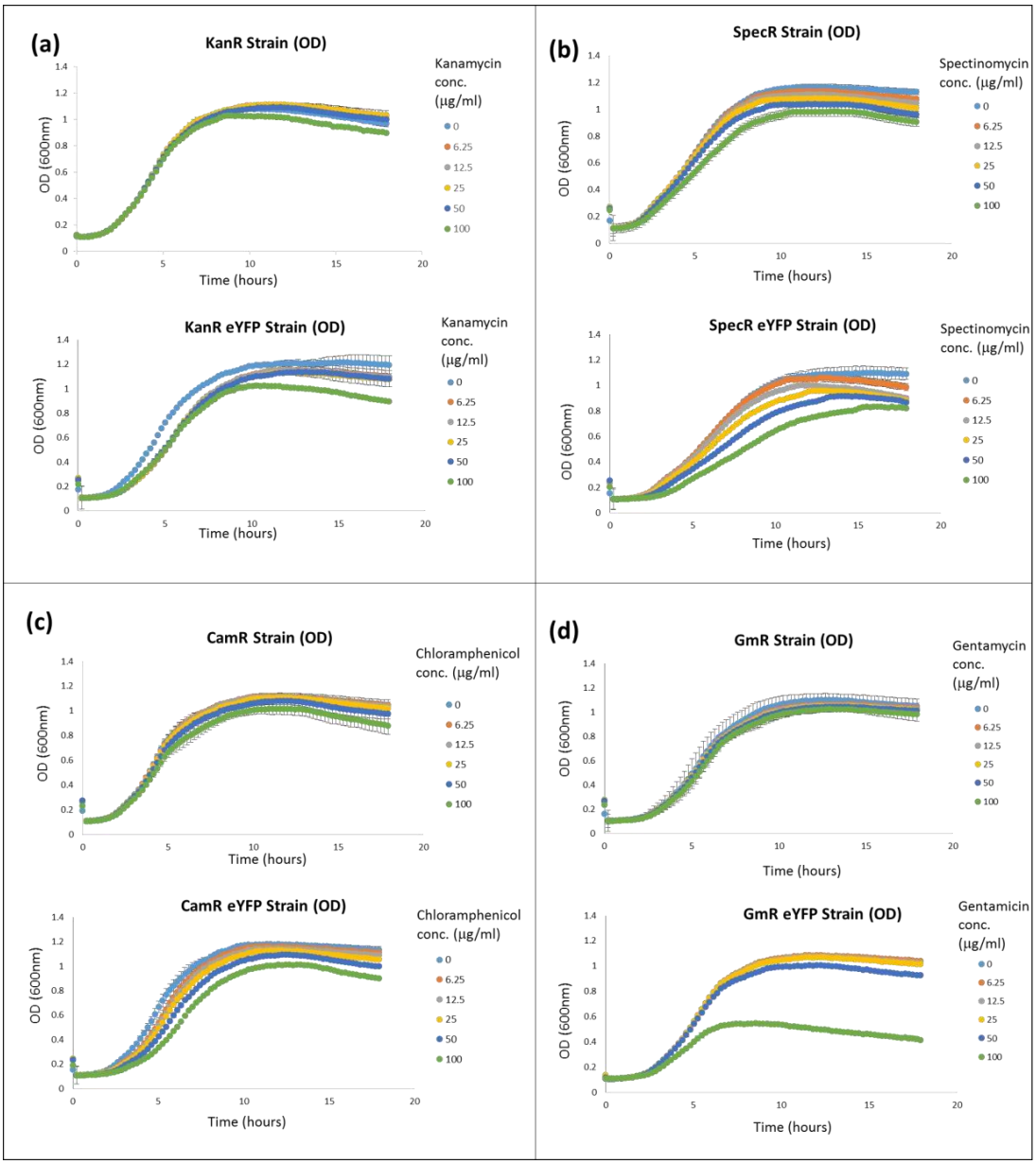

Figure 4. Evaluation of antibiotic resistance capacity of the fusion protein expressing strains. **(a-d)** Showing growth rate comparison between **(a)** KanR and KanR eYFP strains, **(b)** SpecR and SpecR eYFP strains, **(c)** CamR and CamR eYFP strains and **(d)** GmR and GmR eYFP strains, grown in LB media supplemented with 0-100 µg/ml of specific antibiotics. Data shown are the mean of 3 biological replicates. Error bars are ± 1 standard deviation.

b. Fluorescence intensity of expressed tandem fusion proteins

In all the cases, the fluorescence signal reached a maximum after 11 hours of cell incubation which is roughly 4 hours behind to the time when cells reached at their stationary phase (Figure 4, 5).

Cells transformed with KanR eYFP cassette gave a fluorescence signal from 5000-10000 RFU (Figure 5a) whereas, SpecR eYFP carrying cells, no fluorescence was detected (Figure 5b). 7000 to 12000 RFU fluorescence signal was observed from cells transformed with CamR eYFP (Figure 5c). Interestingly, the culture having the lowest OD (grown in 100 µg/ml chloramphenicol) gave the highest signal (12000 RFU) comparing with other cultures grown in presence of chloramphenicol (Figure 5c, 4c). Among all, GmR eYFP strain gave the highest fluorescence signal up to 35000 RFU (Figure 5d), although a significantly lower signal was observed from the cells grown in 100 µg/ml gentamicin, as because the culture OD was also low at that concentration (Figure 5d, 4d).

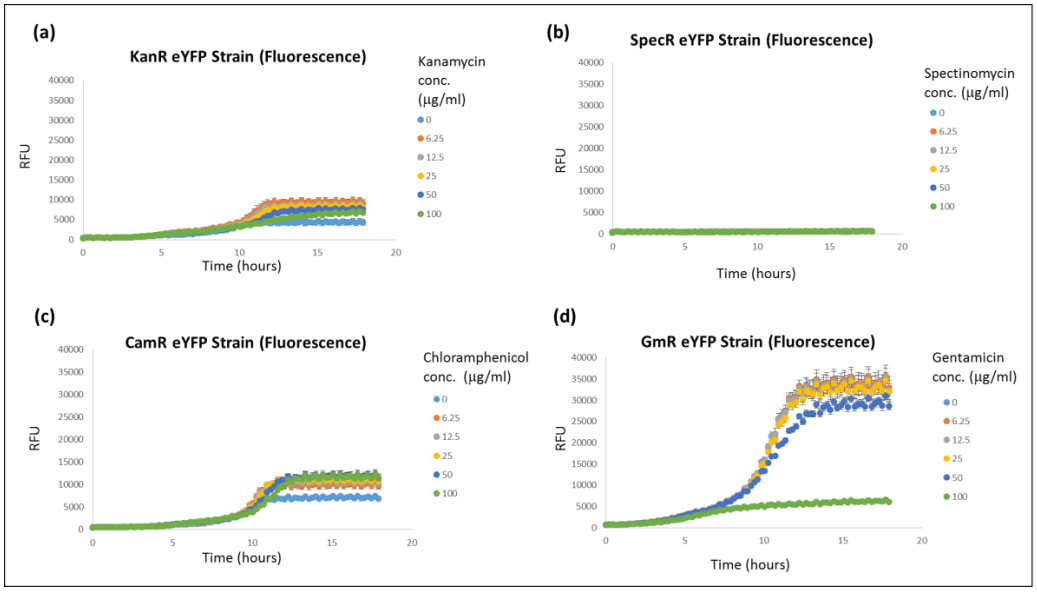

Figure 5. Fluorescence assay of the four fusion proteins. **(a-d)** Showing the signal over time obtained from the cells transformed with **(a)** KanR eYFP cassette, **(b)** SpecR eYFP cassette, **(c)** CamR eYFP cassette and **(d)** GmR eYFP cassette. RFU= Relative Fluorescence Units. Data shown are the mean of 3 biological replicates. Error bars are ± 1 standard deviation.

c. Selection of the best fusion protein

GmR eYFP which was relatively high in expression, was selected to construct the shuttle vector, with the evidence of better antibiotic resistance capacity and high fluorescence intensity (Figure 4d, 5d).

d. Antibiotic susceptibility of wild-type *Synechocystis*

To establish the Ab^R proteins (used in fusion protein construction) as the selection marker for the mutated *Synechocystis*, a susceptibility test of the wild-type *Synechocystis* in the presence of increasing antibiotics concentrations (0-10 µg/ml) was carried out. Growth was completely inhibited by the lowest concentrations (0.625 µg/ml) tested of kanamycin, chloramphenicol and gentamicin (Figure 6a, c, d). However, in 2.5 µg/ml spectinomycin, cells showed noticeable growth, although, growth was completely inhibited in other concentrations (Figure 6b).

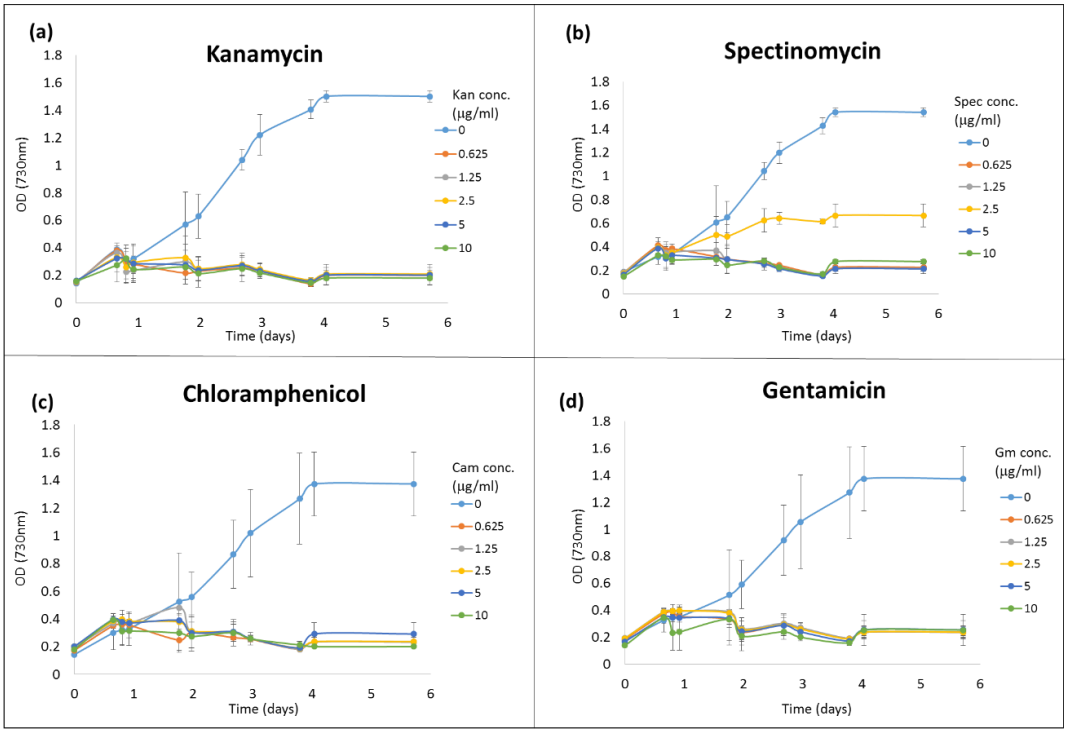

Figure 6. Growth rate assay of wild-type *Synechocystis* at increasing concentration (0-10 □g/ml) of **(a)** Kanamycin, **(b)** Spectinomycin, **(c)** Chloramphenicol and **(d)** Gentamicin. Data shown are the mean of 3 biological replicates. Error bars are ± 1 standard deviation.

e. Construction of shuttle vectors via BASIC Assembly

The shuttles vectors used in this study were previously constructed in the lab targeting two individual native sites of *Synechocystis*- polyhydroxybutyrate (PHB) and glycogen (GlgC) synthesizing region. BASIC assemble by PCR amplification with primers are stated in appendix section (table 3). The presence of desired DNA parts in the backbone was confirmed by PCR using primer "BASIC-Pre-glgCup, BASIC-Suf-glgCdn3" and "BASIC-Pre-PHBup5, BASIC-SufPHBdn3" for GlgC and PHB site targeting vectors, respectively (Appendix II). After electrophoresis gel separation, the amplified DNAs for both the vectors gave band near 4 kb position (Fig 7 a, b).

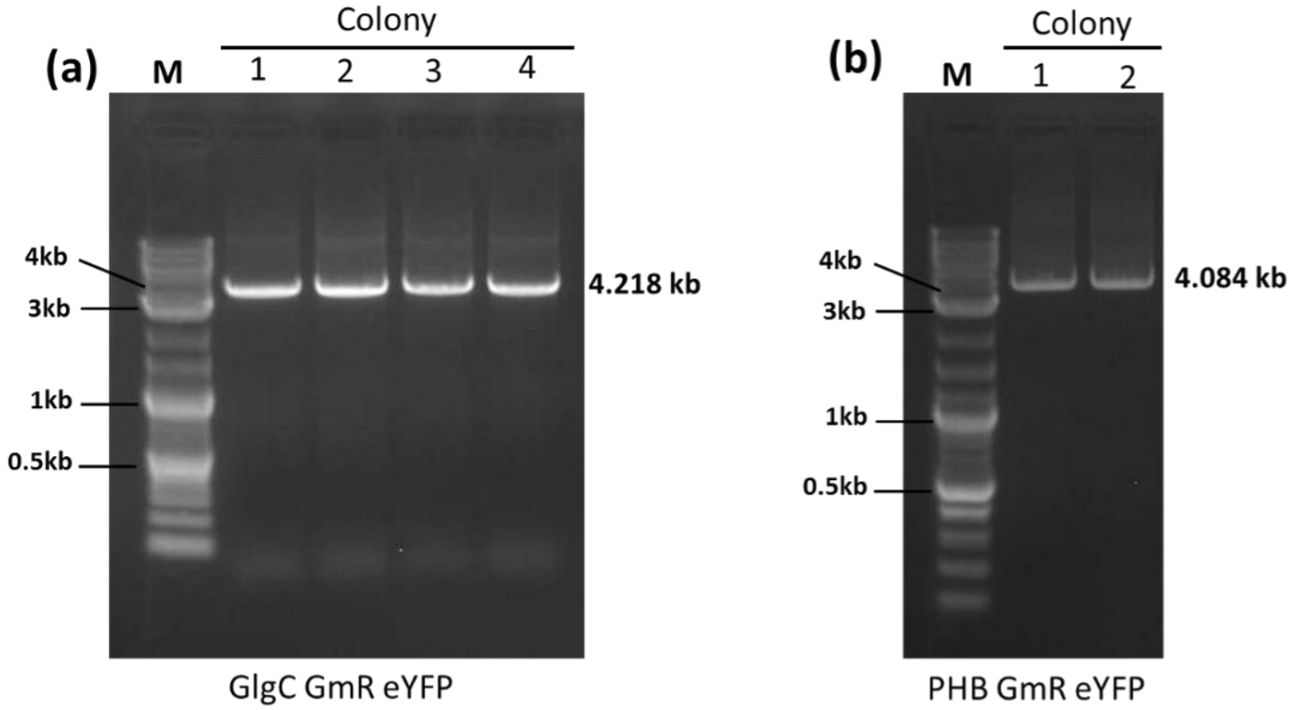

Figure 7. Detection of the presence of fusion cassette with *Synechocystis* 6803 genome derived two DNA parts in the shuttle vector. **(a)** Bands for GlgC GmR eYFP, **(b)** Bands for PHB GmR eYFP. Amplicon size is written on the right side of the gel. M= Marker (2-log ladder).

B. Development of microplate based screening procedure

The efficiency of plate reader based mutant screening was evaluated by mixing the wild type (Wt) *Synechocystis* with a previously engineered Spectinomycin resistant eYFP (CB1-RBS5-eYFP) strain. The experimental setup was stated in the materials and method section.

The selection of mutant cells were carried out in two rounds. In the first round, the culture OD and fluorescence intensity of each of the wells were monitored for 9 days. In presence of spectinomycin, the 100% Wt cultures of row A could not survive, resulting very low fluorescence signal and culture density (Figure 8b, 9a). The cultures of row B and C showed much higher fluorescence and OD, as they started with comparatively higher percentage of mutant cells (0.1% and 0.01% Mut, respectively) (Figure 9a). Contrarily, the cultures of row D (started with 0.001% Mut, theoretically 6 mutant cells/well) initially showed very poor fluorescence and OD, as most of the Wt cells were killed in presence of spectinomycin. However, the theoretical 6 spectinomycin resistant mutant cells of well D2, D3 and D4 doubled over time and showed significant increase in fluorescence and OD after 7 days of incubation (Figure 9a). After the day 9, the well D2 showed the highest recovery of mutant cells with the highest fluorescence intensity, hence, selected for dilution and regrowth (Figure 8b, 9a).

In the second round, the liquid culture of well D2 was diluted in BG-11 medium at OD730 of 0.1, regrown in BG-11 supplemented with 50 □g/ml spectinomycin. After two days of incubation, cultures of the well A1, A5 and B1, B5 were selected (for gene extraction) depending on the higher fluorescence signal with higher culture density (Figure 8c, 9b).

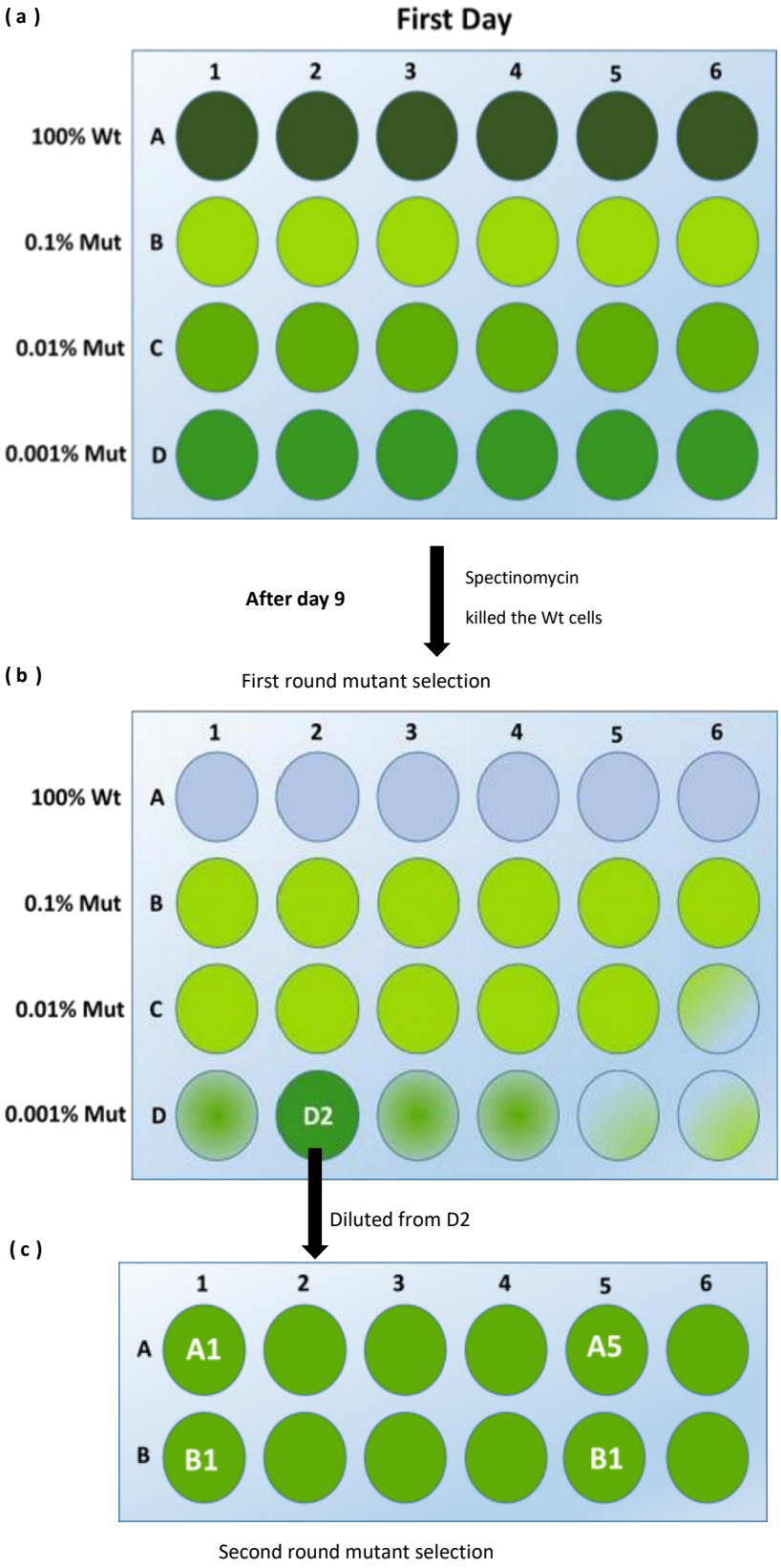

Figure 8. Schematic diagram of the plan for mutant screening via plate reader. Liquid cultures grown in 24 well plates. Wt= wild-type *Synechocystis*, Mut= spectinomycin resistant eYFP strain.

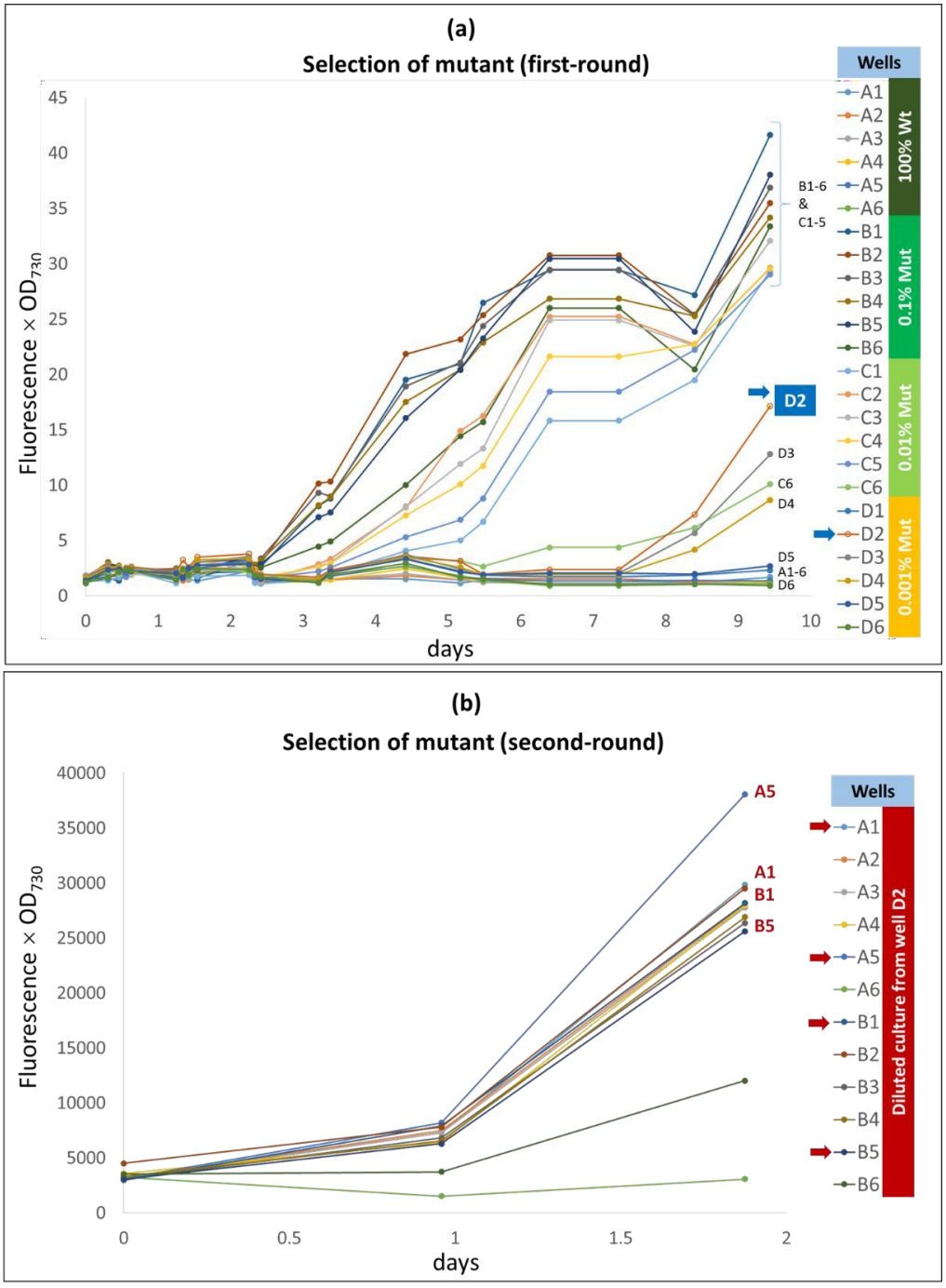

Figure 9. Screening of mutant cells from the mixed culture based on the fluorescence intensity and culture density. **(a)** First round of mutant screening, well D2 (initial 0.001% mutant) was selected for regrowth, with reference of increasing fluorescence and OD (well number is marked with blue arrow). **(b)** Second round of mutant screening. After 2 days incubation, the best performed cultures of well A1, A5 and B1, B5 were selected for genotyping. Difference in Y-axis scale is due to the difference in gain setting used for fluorescence data acquisition.

Following gDNA extraction, cultures were genotyped by PCR using the sequencing primer 74 and 75 (Appendix II). The expected amplicon size of Mut and Wt is 3.84 kb and 200 bp, respectively. After electrophoresis gel run, the gDNA of all the selected cultures gave band near 4 kb position, thus confirming the successful and complete isolation of mutant strain from the mixed culture started with 0.001% Mut (Figure 10).

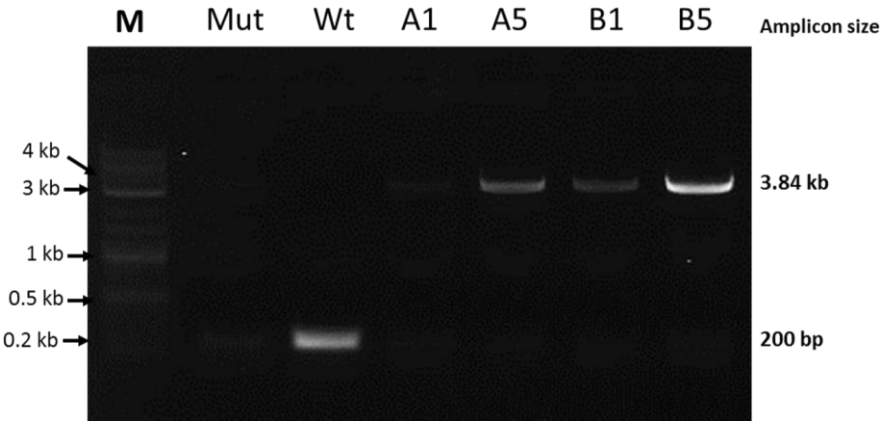

Figure 10. Confirmation of complete isolation of mutant strain from the mixed culture. Amplicon size is written on the right size of the gel. A1, A5, B1, B5 representing the well number of 24 well plate. M= Marker (2-log ladder).

C. Development of FACS-based screening procedure

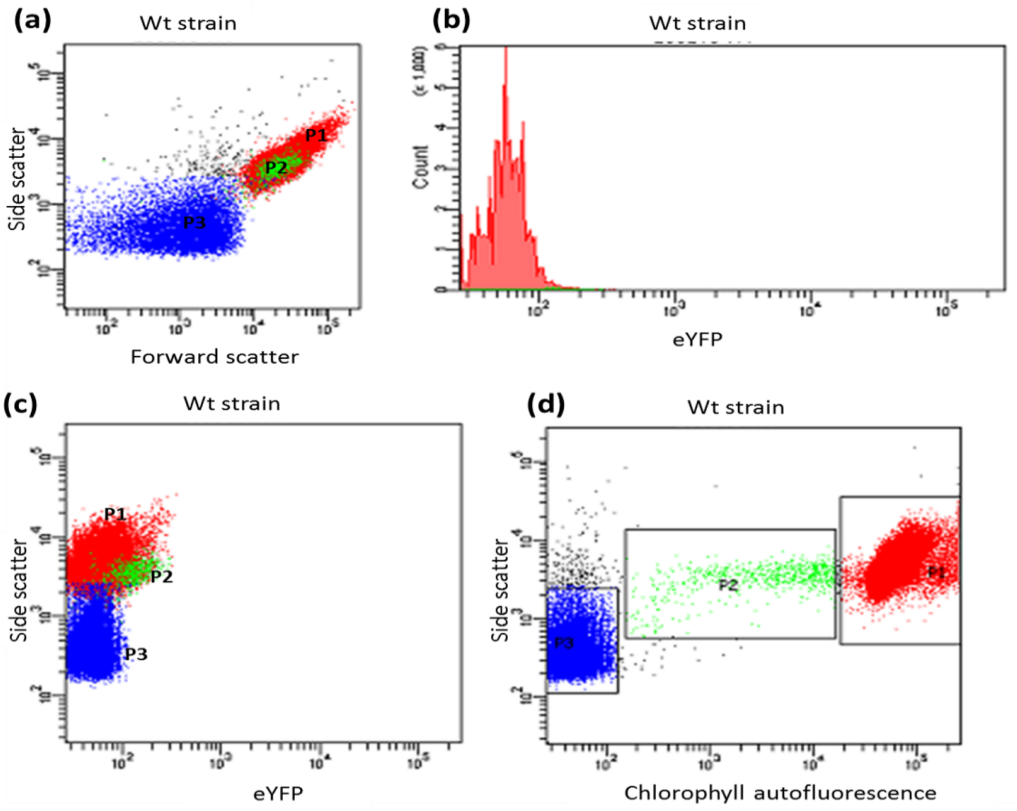

Figure 11. Flow cytometer dot plots of chemically fixed wild-type sample (control). **(a)** SSC vs. FSC showing three individual population- P1, P2 and P3 in the two dimensional dot-plot **(b)** one parameter histogram of eYFP fluorescence (control), **(c)** eYFP panel, low eYFP signal indication, as the sample tested was Wt **(d)** chl autofluorescence panel, P1 showing the highest signal, indicating the viable cells.

Flow cytometric analysis was carried out with samples fixed by 1% formaldehyde. Three cell clusters were observed in two-dimensional dot plot in all the samples. The clusters are marked as P1, P2 and P3 (Figure 11a, 12a and 13a) In the dot plot the population P1 showed much higher intensity in both FSC and SSC than the other two population and the light scattering in FSC and SSC was linearly correlated, therefore, presumed as the viable cyanobacterial cells. In wild-type culture, P1 gave strong signal in chl autofluorescence panel (Figure 13d), confirming this cluster as containing photoautotrophs. In the eYFP panel, all events collected from Wt culture gave very low signal indication, which was expected (Figure 13c).

In case of eYFP (-IPTG) and (+IPTG) cultures, high autofluorescence signal for P1 was detected from the chl autofluorescence channel (Figure 14d, 15d). However, in the eYFP channel there was no shift in the median signal of the population (P1, P2 and P3) in any sample (Figure 12c, 13c).

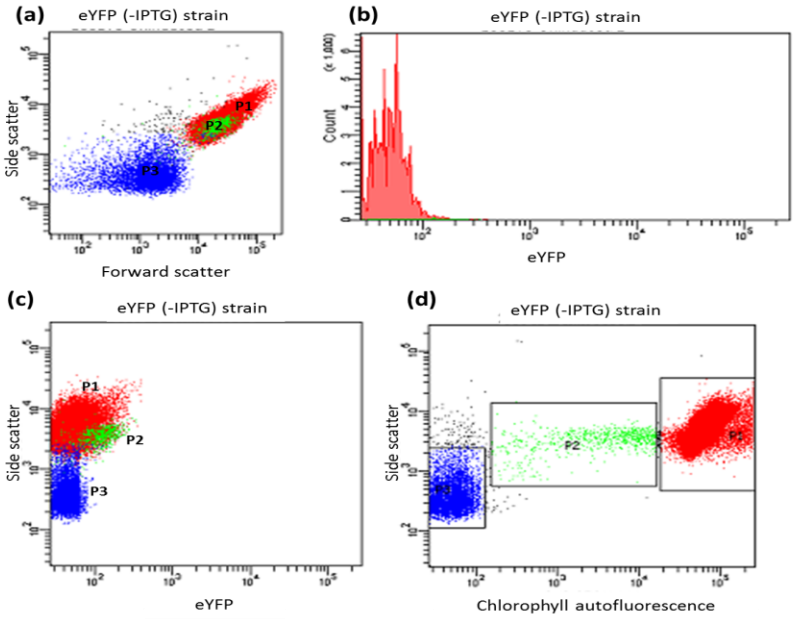

Figure 12. Flow cytometer dot plots of the chemically fixed eYFP (-IPTG) culture. **(a)** SSC vs. FSC showing three individual population- P1, P2 and P3 in the two dimensional dot-plot **(b)** one parameter histogram of eYFP fluorescence, **(c)** eYFP panel, showing no shift in any of the population, **(d)** chl autofluorescence panel, P1 showing the highest signal.

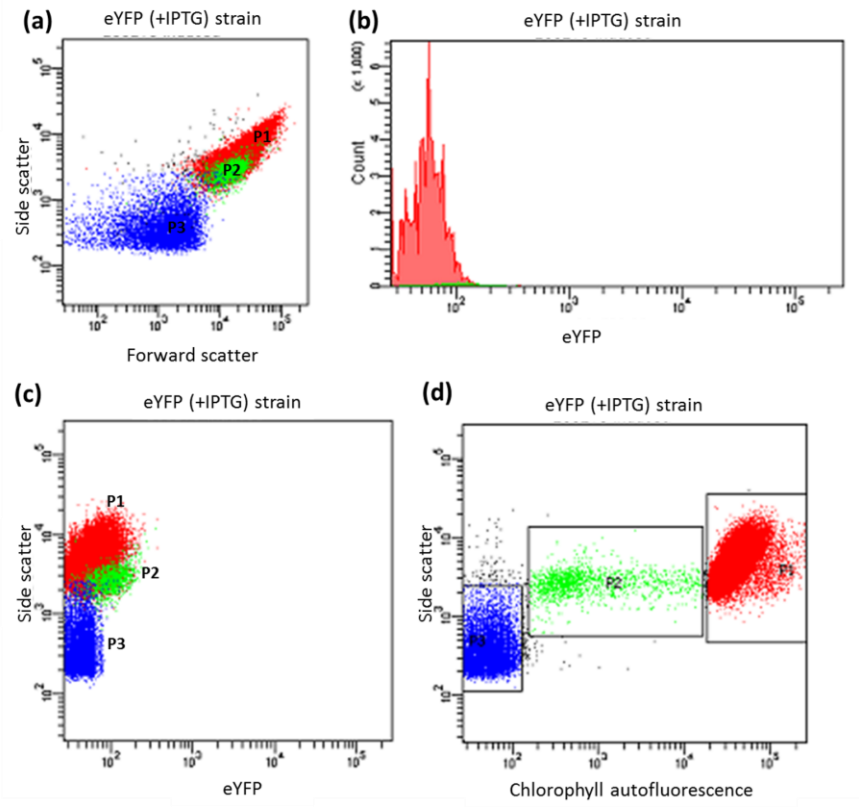

Figure 13. Flow cytometer dot plots of the chemically fixed eYFP (+IPTG) culture. **(a)** SSC vs. FSC showing three individual population- P1, P2 and P3 in the two dimensional dot-plot **(b)** one parameter histogram of eYFP fluorescence, **(c)** eYFP panel, showing no shift in any of the population, **(d)** chl autofluorescence panel, P1 showing the highest signal.

Flow cytometric analysis for eYFP signal detection was again carried out with live (unfixed) cell samples. With respect to granularity and size (SSC vs. FSC) the cells giving high intensity are considered as cyanobacterial cells (78.7% of the total event) and gated in the first dot plot (Figure 14a).

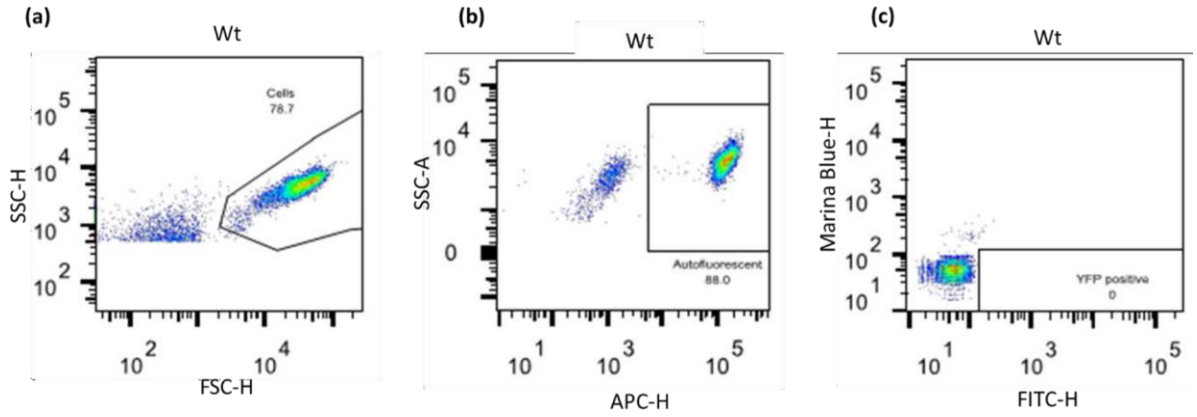

Figure 14. Flow cytometric data acquisition from the live Wt samples (Control). **(a)** Two dimensional dot plot (SSC vs. FSC) showing cyanobacterial population (78.7%) in the total events taken, gated to exclude debris and aggregates **(b)** Gated high chl autofluoresced population (88%) detected in APC channel **(c)** control eYFP panel, logical gating was done to detect eYFP positive cells. The eYFP signal in the FITCH channel is visualized as 2-dimensional dot blots against the Marina Blue-H channel.

In the chl autofluorescence panel, two dimensional plot was set as SSC against APC-H. Based on high chl autofluorescence, a single cell population was gated, which was 88% of the total population (Figure 14b).

In the eYFP panel, Wt cells gave very low signal intensity, which was expected. To detect the eYFP signal from the eYFP positive samples, a logical gating region was selected in the eYFP panel (Figure 14c).

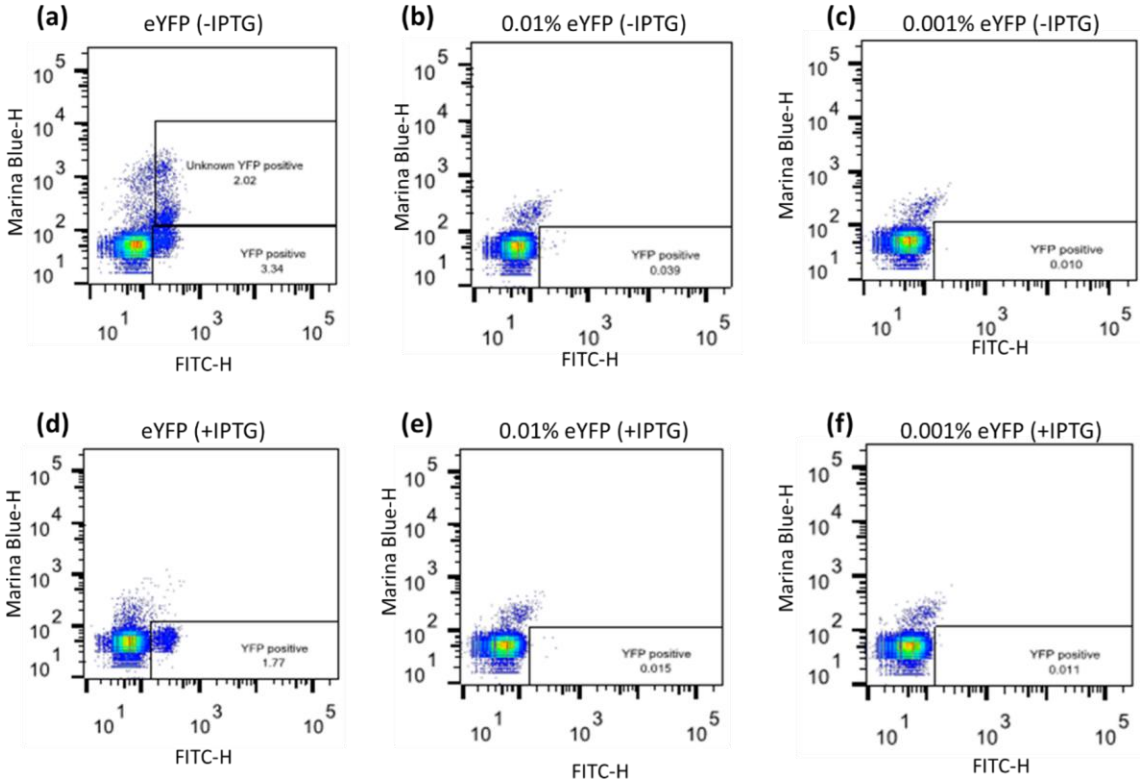

Figure 15. Flow cytometric data acquisition from the live eYFP (-IPTG) and eYFP (+IPTG) samples. **(a-c)** The eYFP events in the gated region for the samples of **(a)** eYFP (-IPTG), **(b)** 0.01% eYFP (-IPTG) cells in mixed culture, **(c)** 0.001% eYFP (IPTG) cells in mixed culture, **(d)** eYFP (+IPTG), **(e)** 0.01% eYFP (+IPTG) cells in the mixed culture and **(f)** 0.001% eYFP (+IPTG) cells in the mixed culture. The eYFP signal in the FITC-H channel is visualized as 2-dimensional dot blots against the Marina Blue-H channel.

In case of eYFP (-IPTG) sample, 3.34% of the population were detected in the gated region (Figure 15a). However, an unknown eYFP population (2.02%) was noticed on the top of the gated region (Figure 17a). In case of mixed cultures (Wt + eYFP cells) containing 0.01% eYFP (-IPTG), 0.039% eYFP events were detected in the gated region (Figure 15b), whereas only 0.01% events were detected from 0.001% eYFP (-IPTG) sample (Figure 15c).

In case of eYFP (+IPTG) sample, 1.77% of the population expressed eYFP signal (Figure 15d). For the mixed cultures carrying 0.01% and 0.001% eYFP cells, the eYFP events were detected as 0.015% and 0.011%, respectively (Figure 15e,f).

Discussion

Fusion protein partners

When constructing the fusion protein, critical selection was made in choosing the biological fluorophore. Cyanobacteria possess fluorescing endogenous pigments, such as- chlorophyll, which is detectable with flow cytometer as strong red or orange autofluorescence (>600 nm) (Hyka *et al.*, 2013). The strong pigment autofluorescence can cause interference of foreign fluorescence protein if it fluoresces within the same spectrum (Hyka *et al.*, 2013). In this project, eYFP - a modified version of GFP was chosen as the fluorescence protein part. The excitation and emission maxima of eYFP is 514 nm and 527 nm, respectively (Shaner *et al.*, 2005). This range does not overlap with the chlorophyll autofluorescence spectrum.

Selection of antibiotic resistance cassettes were made based on the antibiotic sensitivity of *Synechocystis*. The sensitivity test of wild-type *Synechocystis* revealed that, all the antibiotics chosen in this study inhibits the cell growth, hence, well suited as the selection marker.

Bifunctionality of the fusion proteins

Among three successful bifunctional antibiotic resistant eYFP proteins (KanR eYFP, CamR eYFP and GmR eYFP), the GmR-eYFP showed best functionality with respect to both antibiotic resistance (almost similar to the original GmR protein) (Figure 4d) and high fluorescence (at least 3-fold higher than any of the others) (Figure 5d).

In contrast, cells transformed with SpecR eYFP did not give any fluorescence signal (Figure 5b). A report on the successful generation of spectinomycin resistant fluorescent protein with an 11-mer and a 16-mer linker (Khan and Maliga, 1999) demonstrated that, the use of linkers during SpecR eYFP construction may improve its functional efficiency which was absent in the current study. As, a highly functional fusion protein (GmR eYFP) was obtained by tandem fusion, no further effort was given in this project to make a bifunctional SpecR eYFP.

Efficiency of plate-reader based screening approach

The efficiency of plate reader based screening approach is evaluated for the first time in this project. The main idea of this technique is- as plate reader can detect the cellular fluorescence signal, the cells transformed with fluorescence fusion protein can be detected by the plate reader. In the fusion protein, the eYFP CDS was integrated into the AbR cassette. As a result, the eYFP CDS will be transcribed by the native promoter of the AbR cassette that would generate similar expression of these two genes. The higher fluorescence intensity with high resistance capacity will demonstrate the high segregation status of the cell. So the transformed cultures (supplemented with selection agent) that give high fluorescence and higher OD with time (detected by plate reader) can be selected considering as highly segregated mutant cells.

In this project, this screening approach was carried out by mixing the CB1-RBS5-eYFP mutant cells with Wt. The complete segregation of this eYFP mutant strain was already confirmed by another lab member. This experiment was carried out to demonstrate whether the fluorescence from eYFP can be used to enrich and purify of a fully segregated strain out of a population dominated by WT strain (0.001% Mut in the culture) or not. With this approach, complete isolation of mutant strain was confirmed within 11 days.

Flow cytometric analysis

To determine the gating parameter for the eYFP strains, the stability of fluorescence signal from the cells is valuable. As live cells do not provide any guaranty of signal stability, cells were fixed with 1% formaldehyde which caused less alteration in eYFP and Chl autofluorescence. No eYFP signal was observed in fixed cells, although the plate reader data showed only a slight drop in eYFP signal after fixation. Interestingly, the eYFP signal was recovered after using the live cells. This depicts a probability of destroying the eYFP fluorescence by the fixation activity. It is important to note that, flow cytometers analyze the fluorescence from the individual cells, while plate-reader record the average fluorescence in samples (Detection Guide). Although cells were washed thoroughly after fixation, it is still possible that, some fluorophores retained in the liquid media, resulting the eYFP signal in the plate-reader (Jinty et al., 2023).

The eYFP protein can be well-excited with a 488 nm laser (Telford *et al.*, 2012; Lybarger *et al.*, 1998). However, Telford *et al.* (2012) reported that using a laser wavelength more closely matched to the excitation peak (514 nm) helps to achieve a significantly improved sensitivity. As most of the affordable models of flow cytometers are equipped with one blue laser (488 nm) (Hyka *et al.*, 2013), it is strongly recommended to avoid cyanobacterial cell fixation when attempt to visualize the eYFP signal.

Even after assessing the live cells, in the FITC channel, very low eYFP signal was detected and the shift of the eYFP +ve population from the chl autofluoresced population was not distinct enough to select an optimal gating region. Moreover, the percentage of eYFP events detected from the mixed cultures (0.001% eYFP sample) were not linearly correlated with events captured from the 100% eYFP samples. We recommend to increase the total event number to detect a reasonable eYFP events from the 0.001% eYFP mixed culture.

It is also recommended to swap eYFP for GFP may be better suited for commonly available flow cytometers, as it is better matched to the common lasers and filter sets.

Conclusions

In conclusion, it can be said that, the fusion proteins constructed in this study will help to accelerate the engineering of cyanobacteria. The result obtained from the plate-reader based scheme supports the potentiality of the ultimate application after cyanobacterial transformation with fluorescent fusion protein. Moreover, the information gathered from the flow cytometric analysis provided some important information to

improve the design required for further experiment to isolate mutant cells by FACS. Although, this project was focused on the cyanobacteria only, the strategies illustrated in this project can also be employed in the engineering of other polyploid microorganisms, thus could benefit the wider scientific community.

Declaration of Interests

The authors declare that they have no competing interests.

Funding

This work was partially supported by the Centre for Higher Research, Bangladesh University of Professionals and British council.

Acknowledgement

Dr. Paulina Bartasun for her knowledge and support during the study.

Author Contributions

Dr. Mousona Islam and Fateeha Noor designed the overall project. Fateeha Noor and Md. Arifur Rahman Bhuiyan did PCR. Dr. Mousona Islam processed and analysed the sequences and the date. All authors revised the final manuscript.

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