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Investigating Flow Cytometry as a Potential Method for Real-time Analysis of Gene Expression following *Escherichia coli* Transformation

Dallas Bennett, Crystal HT Chau, Ruoyu Ma
Department of Microbiology and Immunology, University of British Columbia

Bacterial transformation is commonly detected by growing colonies on selective media. This process is time consuming and labour intensive. The aim of this study was to develop a more efficient real-time method to observe gene expression following transformation of *Escherichia coli* with plasmid DNA. In this study, we investigated flow cytometry as a potential method for studying transformation. Rather than relying on colony formation to detect transformation, we hypothesised that flow cytometry could be used to detect transformation of single cells shortly after plasmid update. We used pGLO, a plasmid which carries a gene coding for green fluorescence protein gene linked to an arabinose inducible promoter. pGLO was used to transform BL21 *E. coli.* Fluorescence microscopy was used to observe cell morphology and to observe GFP expression over time following induction with arabinose. *E. coli* cells showed morphological changes during increased incubation times. The transformed cells showed green fluorescence 40 minutes after induction with L-arabinose. Using flow cytometry, we were able to detect cells expressing green fluorescence in cultures diluted 10^-4 through to 10^-7, which are expected to have 4000 cells/ml to 4 cells/ml respectively. We were able to detect 3 GFP positive cells in 3180 total cells using flow cytometry. Most notably, newly transformed cells were detected by flow cytometry 1 hour post-transformation. Taken together, these data suggest that flow cytometry can be used to study plasmid transformation and gene expression in real-time. It is also worth noting that variable cell shapes may interfere with measurements; however, this issue may be circumvented by reducing growth time after transformation, with flow cytometry analysis being completed within one hour of transformation.

Bacterial transformation is an important technique in many molecular studies. Transformed bacteria can store genes for future use and be used as a gene expression system. The most common method for bacterial transformation detection is via colony formation in the presence of selective media, generally achieved by plate counting. While plate counting can be time consuming and laborious, flow cytometry could be a more efficient and accurate method to detect transformants in real-time. Flow cytometry discriminates cells based on granularity, size, and the presence of fluorescence tags (1). To date, bacterial transformation assays have not employed flow cytometry as a method to study DNA uptake. Therefore, in this study, we investigated flow cytometry as a method for detecting bacterial transformation. We employed a commercially available plasmid (pGLO) encoding a gene for green fluorescent protein (GFP), which is induced in the presence of arabinose (2). Cells expressing GFP can be counted by flow cytometry (3). Flow cytometry may allow real-time measurements of gene expression following transformation. By comparison, the traditional spread plating method for observing transformation takes about 16 to 24 hours for visible colony growth and may result in satellite colony formation. The objective of this experiment was to develop and optimize a standard detection method of bacterial transformation using flow cytometry.

MATERIALS AND METHODS

Preparation of competent BL21 cells. Before starting competent BL21 cell preparation, 50 ml of TSS solution was made as follows: mixed 5g PEG 8000, 0.30g $MgCl_2*6H_2O$, with 2.5 ml DMSO and then added LB media to a total of 50 ml (4). The TSS solution was then filtered with a 0.22 µm filter and stored at 4°C. BL21 *E. coli* cells were streaked on a LB plate (with no antibiotics) and incubated in an inverted position at 37°C for 16 hours. One isolated colony was inoculated into 20 ml of LB medium and then incubated in a 37°C shaker at 200 RPM. After 2 hours, an OD_{600} reading was taken at 30 minute intervals until the reading reached 0.4. The 10 ml culture was then chilled on ice for 10 minutes and centrifuged at 2700 x g for 10 minutes at 4°C using the Beckman Coulter Avanti J-30I centrifuge. The cell pellet was resuspended in 1 ml of ice-cold TSS solution and aliquoted at 100 µl each into chilled microcentrifuge tubes. All cell aliquots were frozen immediately at -80°C.

pGLO plasmid. pGLO plasmid is a modified pBAD plasmid from Bio-Rad Laboratories Inc (2). It contains a *bla* gene encoding for beta-lactamase, which breaks down ampicillin, a multiple cloning site, an origin of replication, and an *araC* gene, which codes for a regulatory protein that binds to the pBAD promoter. The pBAD promoter is upstream of the GFP gene, so the binding of the regulatory protein allows the induction of GFP expression. A single colony of the pGLO-containing HB101 *E. coli* cells was inoculated in 10 ml of LB-ampicillin media and incubated for 16 hours at 37°C with 200 RPM shaking. Plasmids were purified from the cell culture using the PureLink Quick Plasmid Miniprep Kit (Cat# 205. 10. 11. 01). Nanodrop was used to determine the concentrations of the plasmid before storing the rest of the purified plasmids at -20°C.

PureLink Quick Plasmid Miniprep Kit. Please refer to PureLink Quick Plasmid Miniprep Kit (Cat# 205. 10. 11. 01) handbook from Thermo Scientific.

Transformation of BL21 *E. coli* cells with pGLO. Competent BL21 *E. coli* cells were thawed on ice and then 50 µl of cells were aliquoted for each transformation into pre-chilled 1.5 ml

microcentrifuge tubes. To maintain competency, transformation reactions were kept on ice, unless explicitly stated. Also, all steps in the transformation were performed aseptically to prevent contamination. For each transformation, 50 ng of pGLO DNA was added to the cells and gently mixed with the swirling of the pipet. Each transformation tube was incubated on ice for 10 minutes and then heat shocked in a water bath set at 42°C for exactly 45 seconds without shaking. Immediately after the heat shock, the tubes were placed on ice for 2 minutes to reduce damage to the *E. coli* cells. For the recovery of the cells, 1 ml of LB media was added to each tube and incubated for 1 hour at 37°C (5).

Time course detection of GFP expression. After transformation and recovery of the BL21 *E. coli* cells with pGLO, the transformed cells were grown in LB-ampicillin media at 37°C with shaking for 16 hours. The culture was induced by adding 2 µl of 10% w/v L-arabinose per ml of culture. Immediately after L-arabinose induction, an inoculation loop was used to smear a sample of the induced culture onto a microscope slide. A coverslip was placed on top of the smeared sample, while limiting the amount of bubble formation between the coverslip and the microscope slide.

The microscope slide was observed with the ZEISS Axiostar Plus fluorescence microscope under bright field to locate the cells. Once the cells were located, fluorescence microscopy was used to monitor the cells for GFP expression. The cells at zero time point were compared to a negative control (BL21 *E. coli* cells transformed with pGLO but not induced with L-arabinose). The comparison was performed in terms of morphology and autofluorescence. Images were taken with the 100X oil immersion objective under bright field and fluorescence, starting at 0 minutes and then at 10 minute intervals until a total of 60 minutes.

Flow cytometry. TE buffer pH 8.0 was prepared for use in the BD FACSCalibur and then filtered with a 0.22 µm filter prior to use. An isolated BL21 *E. coli* colony transformed with pGLO plasmid was inoculated into 5 ml of LB-ampicillin media and incubated in the 37°C shaker for 16 hours. Simultaneously, an isolated non-transformed BL21 *E. coli* colony was inoculated into 5 ml of LB media and incubated in the 37°C shaker for 16 hours.

After 16 hours of incubation, both cultures were induced with 2 µl of 10% w/v L-arabinose per ml of culture and incubated for another 16 hours in the 37°C shaker. In order to standardize the number of cells in each sample, the absorbance must be measured at 600 nm after performing a 1:20 dilution to get the culture's turbidity into the accurately detectable limit of the Pharmacia Biotech Ultrospec 3000 spectrophotometer. The rest of the samples, were centrifuged at 2700 x g for 10 minutes at 4°C to pellet the cells. After obtaining the absorbance measurement, the cell pellets were resuspended in TE (tris-EDTA, pH 8) with 1% glutaraldehyde to achieve an OD_{600} reading of 0.05 and fix the cells. The BL21 *E. coli* cells transformed with pGLO were then diluted in TE buffer at the following concentrations to determine the cell concentration that would be optimal for flow cytometry: 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-12}. Once the flow cytometry parameters and optimal cell concentration were determined, the optimal cell concentration was used to perform dilutions of the transformed BL21 *E. coli* cells with the untransformed BL21 *E. coli* cells to run on flow cytometry. Flow cytometry was performed on a FACSCalibur flow cytometer (BD, Sparks, USA) equipped with a 15 milliwatt air-cool argon-ion laser with an excitation wavelength of 488 nm, the major excitation wavelength of GFP, to measure fluorescence and side scattered light. The voltage and flow rate were adjusted for optimal detection at approximately 100-1000 events/second.

Flow cytometry analysis. FlowJo v9.8.1 software was used to analyze the flow cytometry data. Dot plots were produced and gates were hand drawn and the same gates were used within a given data set to allow consistency between the analyses. Populations appear elongated as additional compensation was not applied to the data following collection.

Fluorescence image editing. Grayscale FITC channel images were colorized using Photoshop CC, by converting the image to RBG format and activating the green channel of the RBG channels.

RESULTS

Time course detection of GFP expression following induction. To determine the time required for GFP expression following transformation, we monitored the cells for GFP expression from time=0 minutes to time=60 minutes post-induction using fluorescence microscopy. Following L-arabinose induction of BL21 cultures, very low levels of green fluorescence associated with GFP production was observed for the first 30 minutes. Greater GFP expression was observed at the 40 minute time point, and gradually increased up to 60 minute time point (Figure 1). The high level of green fluorescence was observed at 60 minutes suggests that by one hour post transformation there should be enough fluorescence to analyze the sample using flow cytometry. The cells of the induced culture at the zero time point were the same in terms of morphology and fluorescence as a negative control culture of BL21 *E. coli* cells which were transformed with pGLO but not induced with L-arabinose (Figure 4).

Flow cytometry analysis of mixed fluorescent and nonfluorescent cell populations. The ability of the BD FACSCalibur to differentiate mixed cell populations based on GFP expression must be shown in order to support our method. To do this, two populations of cells: BL21 *E. coli* transformed with pGLO (**GFP(+)**) and non-transformed BL21 *E. coli* (**GFP(-)**), were both induced with L-arabinose. They were then diluted to an OD_{600} of 0.05 with TE buffer. After this, the samples were diluted to 10^-4, which is expected to contain approximately 4000 cells/ml (6). The GFP(+) 10^-4 sample was serially diluted in TE buffer to 10^-5, 10^-6, and 10^-7 and then 0.5ml of each concentration of GFP(+) sample were each mixed with 0.5ml of GFP(-). These samples were run on the BD FACSCalibur (Figure 2). The GFP(-) cell numbers remained relatively constant between flow cytometry runs, which was expected since the concentration of this cell population was not varied in the experiment. Based on Figure 2A, 2B, and 2C, the FACSCalibur detected 3771 GFP(+) and 4359 GFP(-) cells in the two-fold dilution, which are approximately half of the 5157 GFP(+) cells and 8141 GFP(-) cells in the original 10^-4 culture with an estimated concentration of 4000 cells/ml (12). However, the 10^-5, 10^-6, and 10^-7 GFP(+) cells did not follow the expected trend of the ten-fold dilution factor as seen in Figure 2D, 2E, and 2F. Based on the finding that the 10^-4 sample contained 3771 GFP(+) cells, the expected pattern for a 10-fold serial dilution would be 377 cells for the 10^-5 sample, 38 cells for the 10^-6 sample and 4 cells for the 10^-7 sample. However, our flow cytometry results

gave 64, 23, and 14 GFP(+) cells respectively, which was not what was expected. Taken together, these data suggest that it is possible to detect from 14 to 3771 GFP(+) fluorescent

consistent with the time course GFP expression experiment's results. The cell numbers in these samples demonstrated a consistent dose response based on the ten-fold dilution scheme used. The 10^-2 sample had 31 GFP(+) and 31796 GFP(-) cells, which is approximately ten times more than the 3 GFP(+) and 2177

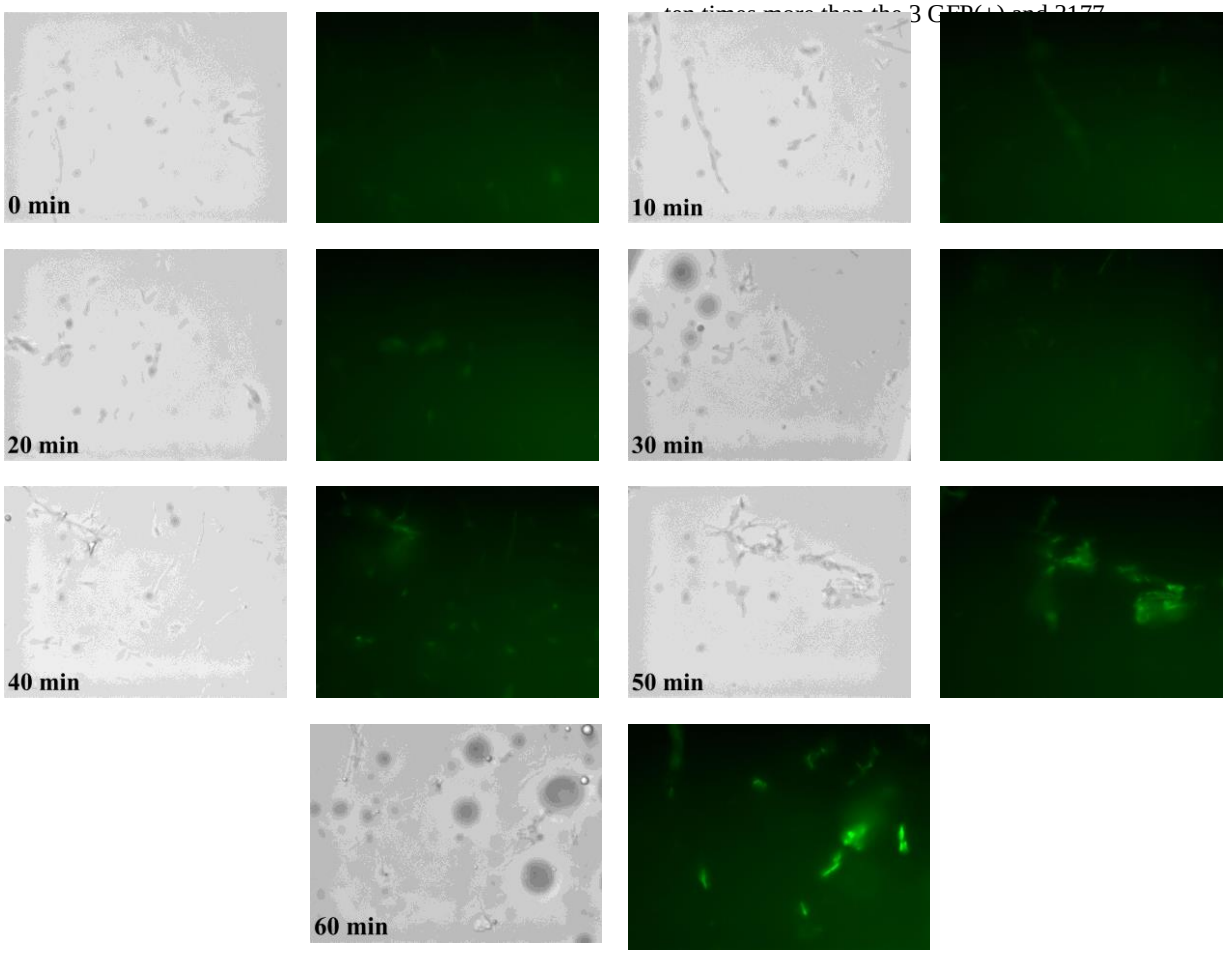

FIG 1 Fluorescence images of GFP expression at 10 minute intervals after L-arabinose induction. Images were taken in 10 minute intervals. Bright field images are included for comparison on the left with fluorescence microscopy on the right. Fluorescence can be observed at 40 minutes with the greatest fluorescence exhibited at 60 minutes post L-arabinose induction.

cells within a greater GFP(-) population of approximately 4000 cells.

Flow cytometry analysis of newly transformed cells. In order to test if cells can be counted on the BD FACSCalibur immediately after transformation, induction with L-arabinose was included during the 1 hour recovery period. This allows for real-time detection of transformants, shortening the time required to screen for transformed cells. A sample of BL21 *E. coli* cells was transformed with pGLO and induced with L-arabinose during the 1 hour recovery period in LB media. Samples were diluted to 10^-2 and 10^-3 and run on the BD FACSCalibur (Figure 3). They were expected to contain 400,000 cells/ml and 40,000 cells/ml respectively (6). There was a population of cells with a detectable level of green fluorescence after 1 hour incubation and induction,

GFP(-) cells in the 10^-3 sample. However, the experimental values were approximately ten times lower than the expected cell numbers. Overall, GFP expression can be detected following the standard process of transformation of E. coli with pGLO using flow cytometry.

DISCUSSION

The goal of this study was to investigate the use of flow cytometry to detect gene expression following *E.coli* transformation. In our experiment, newly transformed cells showed green fluorescence 40 minutes after L-arabinose induction (Figure 1). There are four factors that could contribute to this time delay: the diffusion rate of L-arabinose into the cells, the transcription and

translation time of the GFP gene, the time required for enough accumulation of GFP to be able to detect fluorescence, and the folding time of GFP. In a previous study, the diffusion time of L-arabinose into cells was less than 0.1 minutes, suggesting that the time required for the diffusion of L-arabinose into cells is not likely to

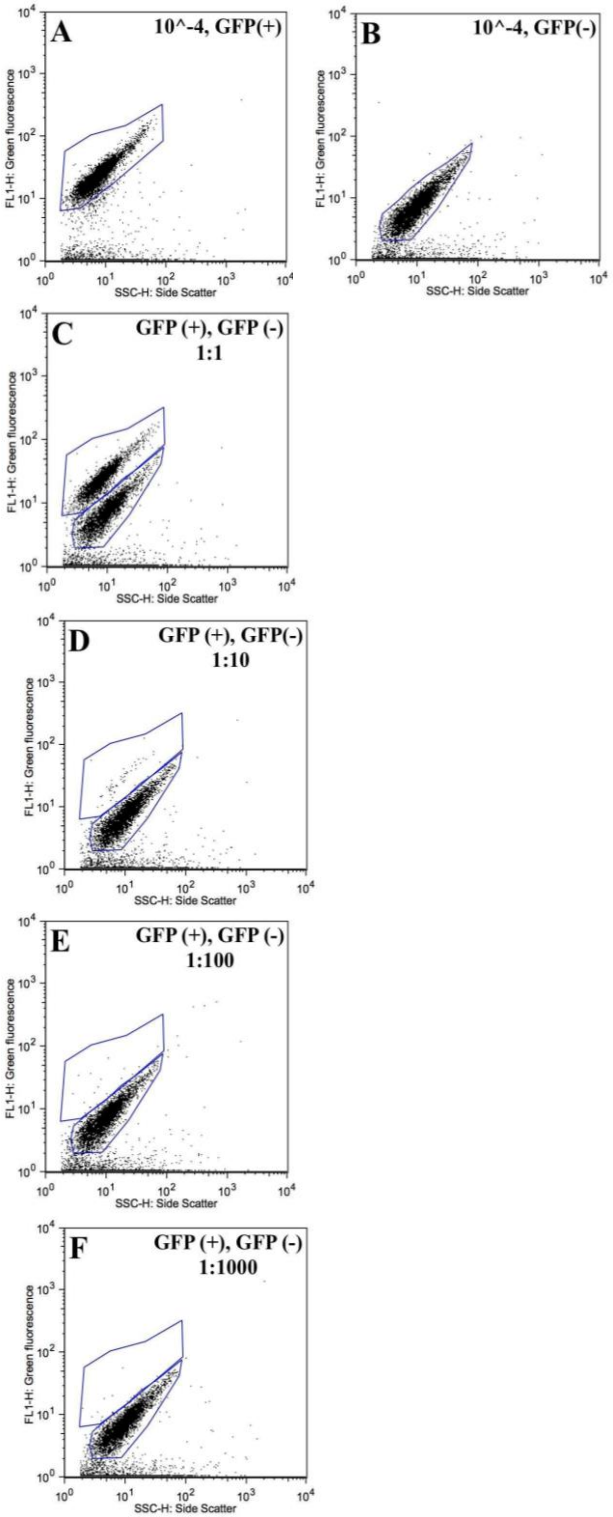

FIG 2 Flow cytometry measurement of serial dilutions of mixed cell populations. Flow cytometry plots of serial dilution samples. The X-axes measure side scattered light, which represents increasing cell granularity while the Y-axes measure increasing green fluorescence, which represents the amount of GFP being expressed. The gating in all the plots were based on the 10^-4 GFP(+) as shown in Figure 2A and on the 10^-4 GFP(-) as shown in Figure 2B. For each run, 1ml of total sample was used. This means that 2C, 2D, 2E and 2F contain 0.5ml of both GFP(+) and GFP(-). *A) 10^-4 GFP(+)* Cell count: 5157. *B) 10^-4 GFP(-)* Cell count: 8141. *C) 10^-4 GFP(+)* Cell count: 3771. *10^-4 GFP(-)* Cell count: 4359. *D) 10^-5 GFP(+)* Cell count: 64. *10^-4 GFP(-)* Cell count: 4514. *E) 10^-6 GFP(+)* Cell count: 23. *10^-4 GFP(-)* Cell count: 4868. *F) 10^-7 GFP(+)* Cell count: 14 *10^-4 GFP(-)* Cell count: 4618.

be a critical factor in the time delay between L-arabinose induction and GFP expression (7). The rate of transcription is 45 to 60 bases per second, suggesting that the transcription time of the GFP gene may not be a rate limiting factor in detection of gene expression (8). In addition, previous studies have shown that the peptide elongation rate observed in *E. coli* cells is 10 to 13 amino acids per second, which suggests that translation of the GFP mRNA is not a major factor contributing to the 40-minute lag from induction to GFP detection (9). GFP is 238 amino acids long and the gene is about 760 bases long (3); therefore, it should take less than one minute to translate one GFP protein. Previous studies have

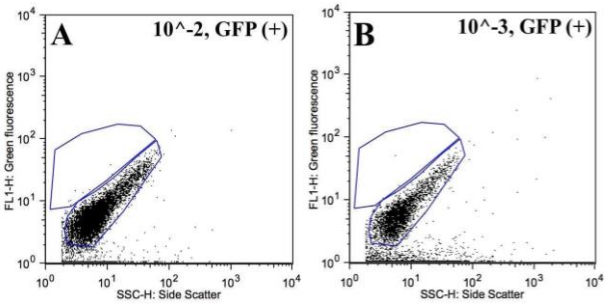

FIG 3 Flow cytometry analysis of an E. coli population 1-hour post transformation with pGLO. Flow cytometry plots of newly transformed cells. The X-axes measure side scatter, which represents increasing cell size while the Y-axes measure increasing green fluorescence, which represents the amount of GFP being expressed. For each run, 1ml of sample was used. *A) Newly Transformed cells, diluted to 10^-2 GFP(+),* Cell Count: 31. *GFP(-),* Cell Count: 31796. *B) Newly Transformed cells, diluted to 10^-3 GFP(+),* Cell Count: 3. *GFP(-),* Cell Count: 3177.

determined that depending on the GFP variant, it takes 30 to 90 minutes for the protein to fold into an operating chromophore (10). From these studies, we suggest that the folding and to a lesser extent, accumulation time required for GFP detection using widefield fluorescence microscopy, are likely the main factors contributing to the 40-minute lag.

To determine the limit of detection of flow cytometry we mixed GFP(+) cells and GFP(-) cells in different proportions. Samples were run on flow cytometry and the lowest cell count in our serial dilution experiment

was 14 GFP(+) cells from a culture with OD_{600} of 0.05 and a 10^-7 dilution factor (Figure 2). The estimated total cell number of this diluted culture is 4 cells/ml, based on the estimate that 1 OD_{600} corresponds to approximately 8x10^8 cells/ml (6). The differences between experimental and estimated cell numbers are within the expected order of magnitude. Discrepancies may be explained by carry over within the flow cytometer from previous runs of GFP(+) cells or simply due to pipetting errors. To verify the accuracy of our data, more replicates of this method need to be performed.

Although BD FACSCalibur could detect from 14 to 3771 GFP(+) fluorescent cells within a greater GFP(-) population of approximately 4000 cells and there is a wide range of detectable concentrations of GFP(+) cells (10^-4 to 10^-7 cells), the accuracy may be decreasing with lower cell concentrations as seen in Figure 2. The cell counts of diluted samples did not demonstrate a consistent dose response to the dilution factor. This could be due to variability error of the machine or human error during pipetting. For quantification, a known spike is likely needed for determination of the capture-rate of flow cytometry.

In conclusion, flow cytometry was used to count cells immediately after transformation. Cells were induced with L-arabinose during the 1 hour recovery after transformation. The BD FACSCalibur counted 31

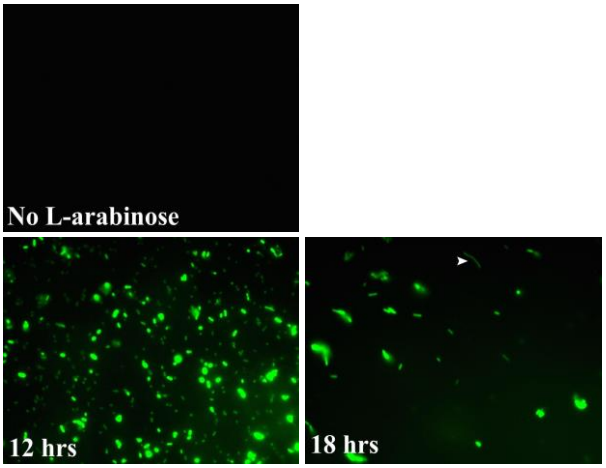

FIG 4 Morphology composition of BL21 *E. coli* cells at 12 hours and 18 hours post induction. BL21 cells did not show any GFP expression without L-arabinose induction. After 12 hours induction, the majority of the cell population showed extensive green fluorescence level and were rod or oblong shaped. In contrast, with 18 hours induction, cells started elongating, some up to 2-3 times as long (see white arrowhead), and several potential aggregates formed.

GFP(+) cells and 31796 GFP(-) cells in a 1:100 dilution (Figure 3). Based on these cell counts, the transformation efficiency is 6.2x10^4 GFP(+) cells/µg. This suggests that flow cytometry can detect GFP expression in BL21 *E. coli* cells 1 hour after transformation with a GFP-containing plasmid. Taken together, these data support the use of flow cytometry as a method to detect gene expression following transformation in real-time. The high sensitivity of flow cytometry could allow experiments to be shortened significantly by removing the requirement of the overnight incubation required for plating methods before determining transformation efficiency.

FUTURE DIRECTIONS

Our results have shown that BL21 *E. coli* cells transformed with pGLO can be detected using flow cytometry and that GFP fluorescence is observed after 40 minutes of induction using fluorescence microscopy. The time course detection of GFP fluorescence was carried out using fluorescence microscopy. A possible extension to the time course experiment would be to utilize flow cytometry to detect GFP fluorescence in a more sensitive manner. Following transformation with pGLO, samples can be induced with L-arabinose for differ lengths of time prior to fixing the cells and counting them on the flow cytometer. This would allow the detection of fluorescence within the whole sample rather than being limited to a small subsample of cells within a specific field of view under the microscope. The sensitivity of flow cytometry at detecting fluorescence compared to the human eye would allow detection of smaller amounts of GFP. This relates to our observation that cells can show diverse shapes within a culture (Figure 4). Flow cytometry may be able to study the cell shape in a mixed population of cells. Cell shape changes during cell growth may impact measurement using flow cytometry however further experiments are required to define this as a possible limitation.

Our results show some variability with respect to the measured limit of detection of this assay method. The number of GFP(+) cells in the 10^-5 sample was 64 (Figure 2D), which was not what was expected from a ten-fold dilution of the 10^-4 sample, which had 3771 GFP(+). This inconsistency to the dilution trend was seen in the 10^-6 and 10^-7 samples as well (Figure 2E and 2F). The unexpected values of the GFP(+) counts at more diluted samples may be due to the detection limit of the FACSCalibur; therefore, it would be beneficial to further investigate the detection limit of the BD FACSCalibur. To begin this process, multiple flow cytometry runs with a range of sample dilutions are needed to explore the reproducibility and accuracy of the data.

Additionally, we have only investigated the detection of GFP expression in BL21 *E. coli* cells transformed with pGLO. Different plasmids containing GFP should be tested, in order to account for the different GFP expression levels and time required for induction. For instance, if a pBR322 was used as a vector for GFP, then the production of GFP would be driven by a T7 promoter that is induced by IPTG. This would likely alter the induction time required for GFP expression from our results of 40 minutes. Also, the use of recombinant genes, marking the gene of interest with the GFP gene, could allow the expression of these target genes to be monitored by flow

cytometry. The information gained from these experiments could allow flow cytometry to be developed into a method of analysis for expression of other genes during transformation studies.

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