Biochemistry

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Fluorescence Polarization Assay for Small Molecule Screening of FK506 Biosynthesized in 96-Well Microtiter Plates

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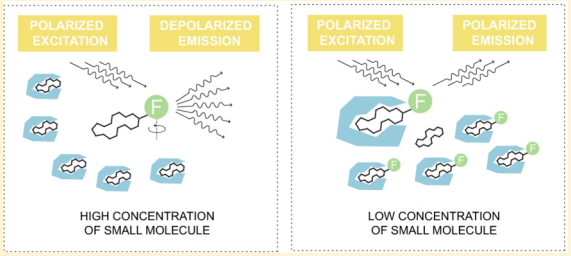
Supporting Information

ABSTRACT: The fluorescence polarization (FP) assay has been widely used to study enzyme kinetics, antibody−antigen interactions, and other biological interactions. We propose that the FP assay can be adapted as a high-throughput and potentially widely applicable screen for small molecules. This is useful in metabolic engineering, which is a promising approach to synthesizing compounds of pharmaceutical, agricultural, and industrial importance using bioengineered strains. There, the development of high-yield strains is often a costly and time-consuming process. This problem can be addressed by generating and testing large mutant strain libraries. However, a current key bottleneck is the lack of high-throughput screens to detect the small molecule products. The FP assay is quantitative, sensitive, fast, and cheap. As a proof of principle, we established the FP assay to screen for FK506 (tacrolimus) produced by *Streptomyces tsukubaensis*, which was cultivated in 96-well plates. An ultraviolet mutagenized library of 160 colonies was screened to identify strains showing higher FK506 productivities. The FP assay has the potential to be generalized to detect a wide range of other small molecules.



Metabolic engineering has the potential to revolutionize the production of pharmaceuticals, chemicals, and fuels by lowering its cost and environmental impact, as well as providing access to novel compounds.[1,2] However, successful examples such as engineering yeast to produce the antimalarial precursor amorphadiene[3] and *Escherichia coli* to produce the bulk chemical 1,3-propanediol[4] all required heroic effort, time, and cost to achieve. This is understandable given that metabolic engineering is a combinatorial optimization problem involving multigene pathways and interacting cellular networks.[5] As such, platforms that enable the high-throughput creation and screening of large populations of mutant strains can transform the speed, cost, and scale of metabolic engineering. Although many technologies have been established to generate large strain libraries (>10^6),[6] a current bottleneck in applying combinatorial approaches to metabolic engineering is the lack of generalizable, high-throughput screens for small molecule targets.[7−10]

The current state of the art for small molecule detection is gas/liquid chromatography−mass spectrometry (GC/LC−MS), which is an expensive and low-throughput method (~10^2 samples per day).[7] High-throughput methods such as FACS or growth selections (~10^7 samples per day) require that the target molecule be colored,[11] or be fluorescent,[12] contain reactive groups,[13] or confer a growth advantage on the producer cell[14] and thus cannot be generalized to the majority of targets. Recently, assays that rely on adapting natural regulators such as riboswitches,[15] transcription factors,[16] and nuclear hormone receptors[17] have been developed. However, there have been few reports of their successful redesign or evolution to change their target specificity,[18,19] so their generality is still unknown. Likewise, receptors that are labeled directly with fluorescent proteins,[20,21] enzymes,[22] environmentally sensitive fluorophores,[23−25] and RNA/DNA molecular aptamer beacons or aptasensors[26] have yet to be widely adopted. This is because their binding and signal transduction functions are tightly interwoven, which makes it difficult to alter target specificity, yet have the receptors keep their signaling function.

We propose that the fluorescence polarization (FP) assay, when applied as a competition assay, can offer a broadly applicable and high-throughput screen for small molecule targets. Although the FP assay has been used for drug discovery and to study enzyme kinetics, antibody−antigen interactions, and other biological interactions,[27,28] its potential for small molecule detection for metabolic engineering remains un-

Received: June 26, 2017
Revised: August 6, 2017
Published: August 25, 2017

DOI: 10.1021/acs.biochem.7b00602
Biochemistry 2017, 56, 5260−5268

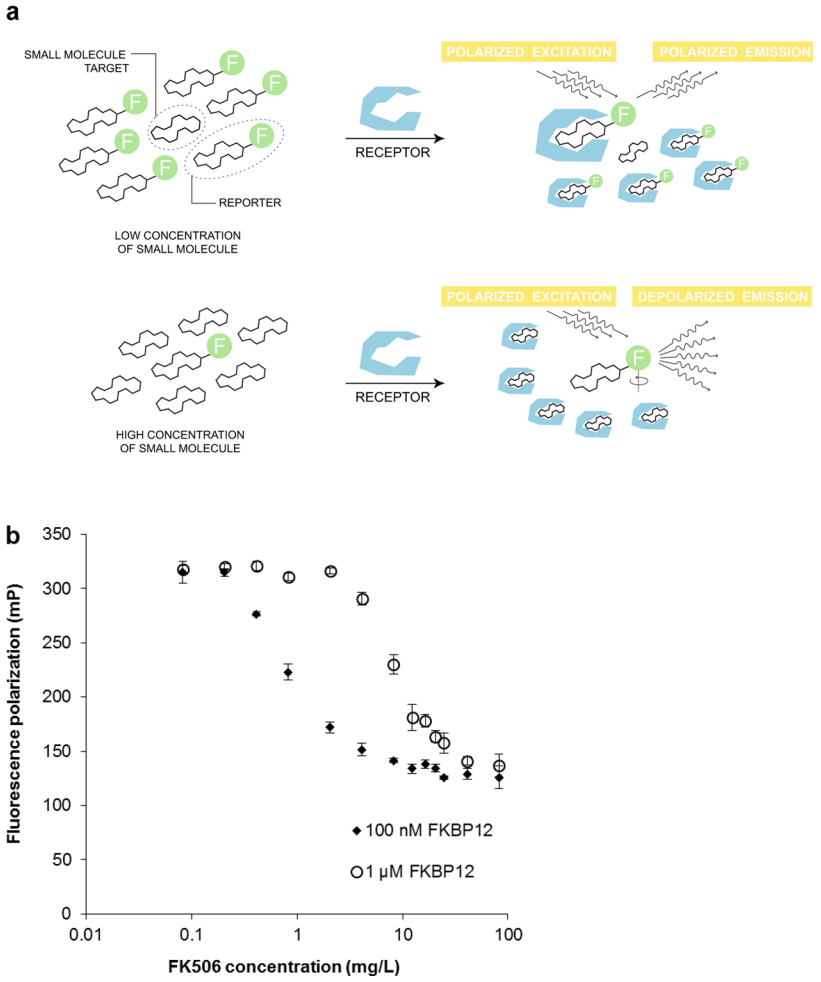


Figure 1. FP assay for detecting FK506. (a) Principle of applying the FP assay to detect small molecules. (b) Standard curves for commercial FK506 using 100 nM and 1 μM receptor FKBP12. Error bars are standard deviations of three readings each of three replicate wells. Concentrations refer to that of the added sample. The final concentration in the assay well is 10-fold lower.

tapped. The assay requires two components: a target receptor, which can be derived from nature or by directed evolution,[29] and a reporter, which can be made by coupling the target molecule to a fluorophore. The target molecules compete with the reporter for binding to the common receptor. Thus, the target concentration is inversely proportional to the ratio of bound to unbound reporter molecules, which can be measured by FP (Figure 1a).

As a proof of concept, we have established the FP assay as a high-throughput screen for the polyketide FK506, which is naturally produced by *Streptomyces tsukubaensis*.[30,31] FK506 (tacrolimus) is the major immunosuppressive drug used to prevent the rejection of organ transplants (e.g., kidney, liver, and heart) and also used topically to treat atopic dermatitis, with annual sales in the United States alone approaching $1 billion.[32] It functions by binding to the intracellular receptor FKBP12, which triggers a signaling pathway leading to immunosuppression.[33] Because of the off-patent status of tacrolimus as well as a need to reduce off-target effects, there is a strong interest in strain and culture process development to optimize the production of the drug and its analogues.[34−36]

Previously, FP assays using the receptor FKBP12 and various fluoresceinated reporters have been developed to identify novel FKBP12 ligands,[37] to study FK506-binding proteins such as FKBP51 and FKBP52,[38] and to measure the affinity of FKBP12 receptor mutants for FK506.[39] In this study, we have adapted the FP assay to screen for FK506 production by *S. tsukubaensis* cultivated in 96-well, deep-well microtiter plates. The FP assay for FK506 was performed using the receptor FKBP12 [purified using the Ni-NTA kit (Qiagen) from a previous construct[39]] and reporter molecule FK506-fluorescein (a gift from Ariad Pharmaceuticals).[39,40] The assay was performed in 384-well, round, black-bottom plates (Corning 3821). A total volume of 40 μL per well was used. The final concentrations of reagents in each well were 2.5 nM FK506-fluorescein and 1 μM FKBP12 (unless otherwise specified). The order of addition of each reagent to the well was as follows: 32 μL of master mix containing FK506-fluorescein, 2× FP assay buffer,[39] and water, then 4 μL of the sample (in methanol solvent), and then 4 μL of 10 μM FKBP12. The plates were incubated at room temperature for 2 h before readings were taken using a Victor X5 plate reader (PerkinElmer), with a 485 nm excitation filter and a 535 nm emission filter.

A FP standard curve was constructed using commercial FK506 standards purchased from Sigma (F4679). The shape of the curve was sigmoidal, with the FP readings approaching a maximum at high FK506 concentrations (this is determined by the affinity of the reporter for the receptor when the assay is performed at equilibrium and the receptor is not limiting) and a minimum at low FK506 concentrations (the baseline is caused

DOI: 10.1021/acs.biochem.7b00602
Biochemistry 2017, 56, 5260−5268

by nonspecific binding of the reporter to other components of the assay or the walls) (Figure 1b). We performed the FP assay at two different receptor concentrations (100 nM and 1 μM), which gave detection ranges of ~0.1−10 and ~1−20 mg/L FK506 respectively (Figure 1b).

Although the dynamic range of product detection is limited for a particular receptor concentration, this range can be easily tuned by changing the receptor concentration. This principle can be used to adjust the level of stringency in a high-throughput screen. For example, the receptor concentration can be increased over multiple rounds of screening to detect higher target concentrations. Alternatively, the samples can be diluted or concentrated such that the target concentrations fall within the dynamic range of the assay. The lower limit (sensitivity) of the assay depends on the K_d of the target and reporter molecules, the receptor and reporter concentrations used, and the nature of the fluorophore. For molecules with K_d values in the low nanomolar range, it should be possible to detect a compound concentration as low as 10 nM by decreasing the receptor concentration (e.g., 50 nM). The concentration of reporter molecules used in this study was 2.5 nM, which has been previously optimized[39] and is the minimum required for a reliable FP signal. For other FP assays, the minimum concentration of reporter molecules can be empirically determined by testing various combinations of receptor and reporter concentrations, until a reliable difference in the FP signal between the presence and absence of the receptor is observed.

For the FP assay to be useful as a high-throughput screening platform for metabolic engineering, the production platform has to be of similar throughput and compatible with the microtiter plate format used in the FP assay. Thus, we sought to produce FK506 by cultivating *S. tsukubaensis* (NRRL 18488) obtained from the Agricultural Research Service Culture Collection (NRRL) in 2.2 mL, square, 96-well deep-well plates (Axygen Scientific). Microtiter plates are cheap, automatable,[41] reproducible, and high-throughput compared to shake flasks.[42,43] However, there have only been a few reports of culturing filamentous organisms such as *Streptomyces* spp. in multiwell plates.[42−45] This is because oxygen transfer rates in microtiter plates can quickly become limiting for cell growth and secondary metabolite production. This can be overcome with the use of high-frequency shakers, smaller culture volumes, square wells, tilted rotation, and larger shaking orbits.[42] While rapamycin has been produced in a 96-well plate format,[43,45] to the best of our knowledge, the production of FK506 in liquid culture in 96-well microtiter plates has not yet been reported.

We cultivated *S. tsukubaensis* in three different volumes of media (500, 750, and 1000 μL) and tested for the production of FK506 over the course of 8 days. A modified two-stage cultivation method was used;[31] 500 μL of modified BaSa seed medium was inoculated with NRRL 18488 spores and incubated for 3 days at 28 °C and 250 rpm (Innova 44R, 2 in. orbit). The seed culture (10%) was used to inoculate 500−1000 μL of ISPz medium[31] and incubated for 4−8 days at 28 °C and 250 rpm. All plates were sealed with two layers of a sterile, breathable rayon seal (AeraSeal). The contents of each well was extracted separately by first transferring it into a 2 mL Eppendorf tube. An equal volume of ethyl acetate was added, and the tube was vortexed for 30 min. After centrifugation for 5 min, the top ethyl acetate layer was transferred to a 4 mL glass vial and evaporated to dryness by rotary evaporation. An equal volume of methanol was added to dissolve any FK506 present for subsequent testing.

The amount of FK506 produced was quantified by both mass spectrometry and the FP assay. Mass spectrometry was performed using an LC−MS instrument (Agilent 1100 Series Capillary LCMSD Trap XCT Spectrometer, New York University). A standard curve was generated by selected ion monitoring of ion counts at m/z 826.4 (FK506 + Na) using different concentrations of commercial FK506 (Sigma F4679). This curve was used to determine the concentration of FK506 in the sample.

Production of FK506 was observed after 24 h for all three volumes and increased over the course of 8 days (Figure 2a). Production levels as measured by the FP assay reached ~15 mg/L after 8 days for both 750 and 1000 μL, which was higher than that for 500 μL (~11 mg/L) (Figure 2a). We compared the amount of FK506 in all our samples as measured by mass spectrometry and the FP assay and found that the amounts correlate (R^2 = 0.705) (Figure 2c). Significantly, the FP assay displayed variability much lower than that of the mass spectrometry measurements, as evidenced by the smaller error bars and by application of the F-test (Figure 2c and Table S1). Next, we measured cell growth using the dry weight method, taking into account submerged mycelia as well as growth on the walls above the liquid level (Figure 2b). The cells were pelleted in preweighed 4 mL glass vials, washed twice with acid (pH = 1.0) to remove insoluble $CaCO_3$ in the medium, and then dried in an oven for 2 days before the mass was measured. Large error bars were observed for 144, 168, and 192 h, which was due to the challenge of recovering all cell clumps/mycelia. Thus, we have subsequently adopted a methylene blue assay that has a lower variability and is amenable to automation (Figure 3b).

FK506 is insoluble in water and is not secreted from the producer cells, which necessitates an additional extraction step. Previously, the contents of each well was transferred individually into microcentrifuge tubes for ethyl acetate extraction. However, this process is labor-intensive and is not practical for screening libraries. Thus, we developed a protocol for extracting FK506 from the *Streptomyces* cells within the 96-well production plates, which is amenable to handling with a multichannel pipet. First, the cells in the plates were subjected to three freeze−thaw cycles; 100 μL of culture was used for the methylene blue assay. An equal volume of ethyl acetate was added to the remaining culture. The plates were sealed with a silicone mat (ImpermaMat, Axygen Scientific), wrapped with parafilm, and incubated for 1 h at 20 °C and 800 rpm in a high-frequency plate shaker. The plates were then centrifuged for 5 min at 3000 rpm; 100 μL of the top ethyl acetate layer was gently transferred to a 96-well polypropylene plate (Greiner). The plate was evaporated to dryness in a chemical hood, and 100 μL of methanol was added to dissolve the extracted FK506. This was added directly into the assay wells for the FP assay. The protocol was tested by spiking a series of FK506 standards into production medium without cells and performing the extraction within the plate. We then measured the extracted standards with the FP assay, which matched the results obtained from standards that were directly added to the FP assay (Figure 3a). The recovery ratio of FK506 from medium over a range of FK506 concentrations is close to 100% (Table S2). Performing the extraction step in 96-well plates would allow for potential future automation of the extraction step,

DOI: 10.1021/acs.biochem.7b00602
Biochemistry 2017, 56, 5260−5268

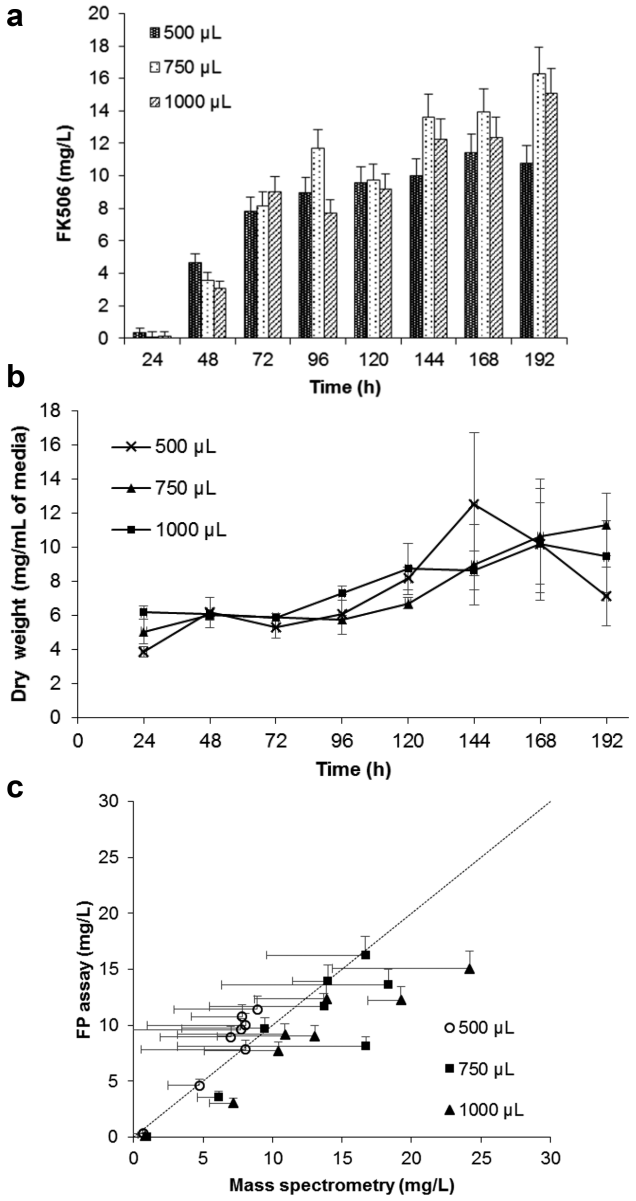


Figure 2. Production of FK506 in 96-well plates. (a) FK506 produced (in milligrams per liter of production medium) over 8 days using 500, 750, and 1000 μL per well, as measured by the FP assay. All values were background subtracted (i.e., from wells containing the ISPz medium alone). (b) Growth curves of cells for 500, 750, and 1000 μL, as measured by the increase in dry mass over time. (c) Correlation chart for mass spectrometry and FP measurements. For the sake of clarity, only the left error bars (mass spectrometry measurements) and top error bars (FP measurements) are shown. All data points are the means of four wells, and all error bars represent the standard deviation from four wells.

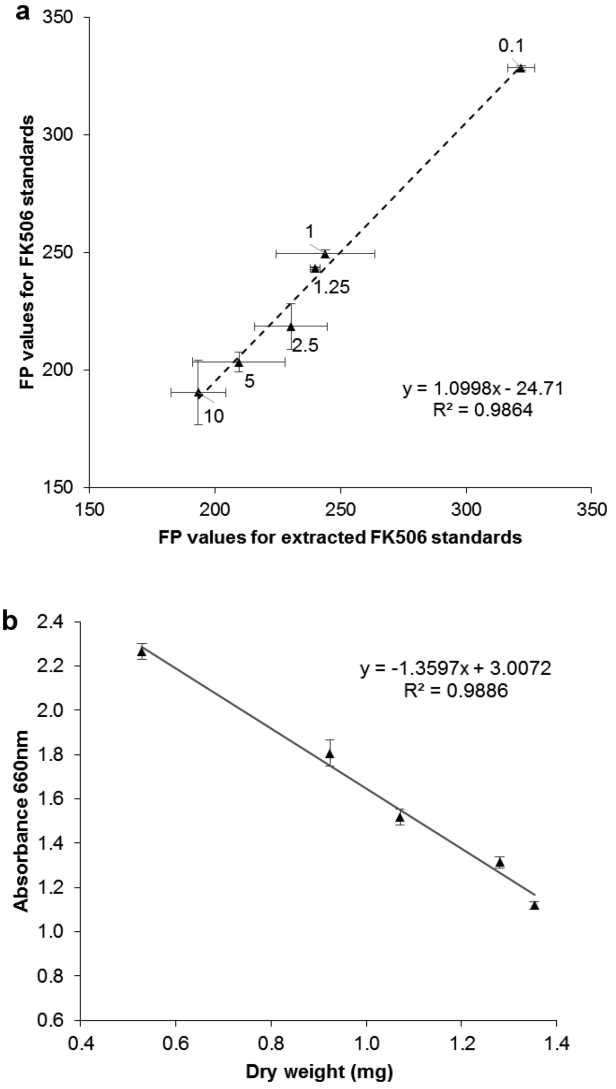


Figure 3. Extraction of FK506 and growth determination in 96-well plates. (a) Plot of FP values for non-extracted FK506 standards vs extracted FK506 standards (in micromolar) extracted from medium in 96-well plates. Error bars are the standard deviation of three readings each of two replicate wells. (b) Standard curve of methylene blue absorbance (at 660 nm) vs the dry weight of cells.

which should increase the throughput and reproducibility of the data obtained.

We also adopted the methylene blue assay to quantify the growth of *S. tsukubaensis* cells in 96-well plates;[46] 100 μL of a methylene blue solution (final concentration of 1.5 mM) was added to 100 μL of sample in a Corning Costar polystyrene clear 96-well plate. The plate was incubated for 15 min at 80 °C, and the contents were mixed every 5 min in a high-frequency microplate shaker (800 rpm, 10 s). The plate was centrifuged for 5 min at 3000 rpm, and 10 μL of the supernatant was mixed with 190 μL of water in a new plate. The absorbance was determined in a Tecan plate reader at 660 nm. Using a series of samples with different amounts of cells, we constructed a standard curve of methylene blue absorbance against dry weight (Figure 3b). The curve shows a linear relationship with the amount of methylene blue left unabsorbed in solution decreasing proportionally with an increasing amount of cells. This method requires only a small aliquot of the cell suspension and is rapid (~30 min) and high-throughput compared to dry weight measurements, which may require more than a day for the sample to dry in an oven.

To illustrate a practical application of our plate production and FP screening platform, we screened an ultraviolet (UV)-mutagenized library of 160 *S. tsukubaensis* colonies for FK506 production (Figure 4a). NRRL 18488 was grown on inorganic salts/starch agar medium (ISP4) (BD Difco, 277210) at 28 °C for 4 days to obtain spores. These were harvested, and the spore suspension was irradiated with UV light using a Stratalinker 2400 instrument (Agilent) calibrated to obtain a

DOI: 10.1021/acs.biochem.7b00602
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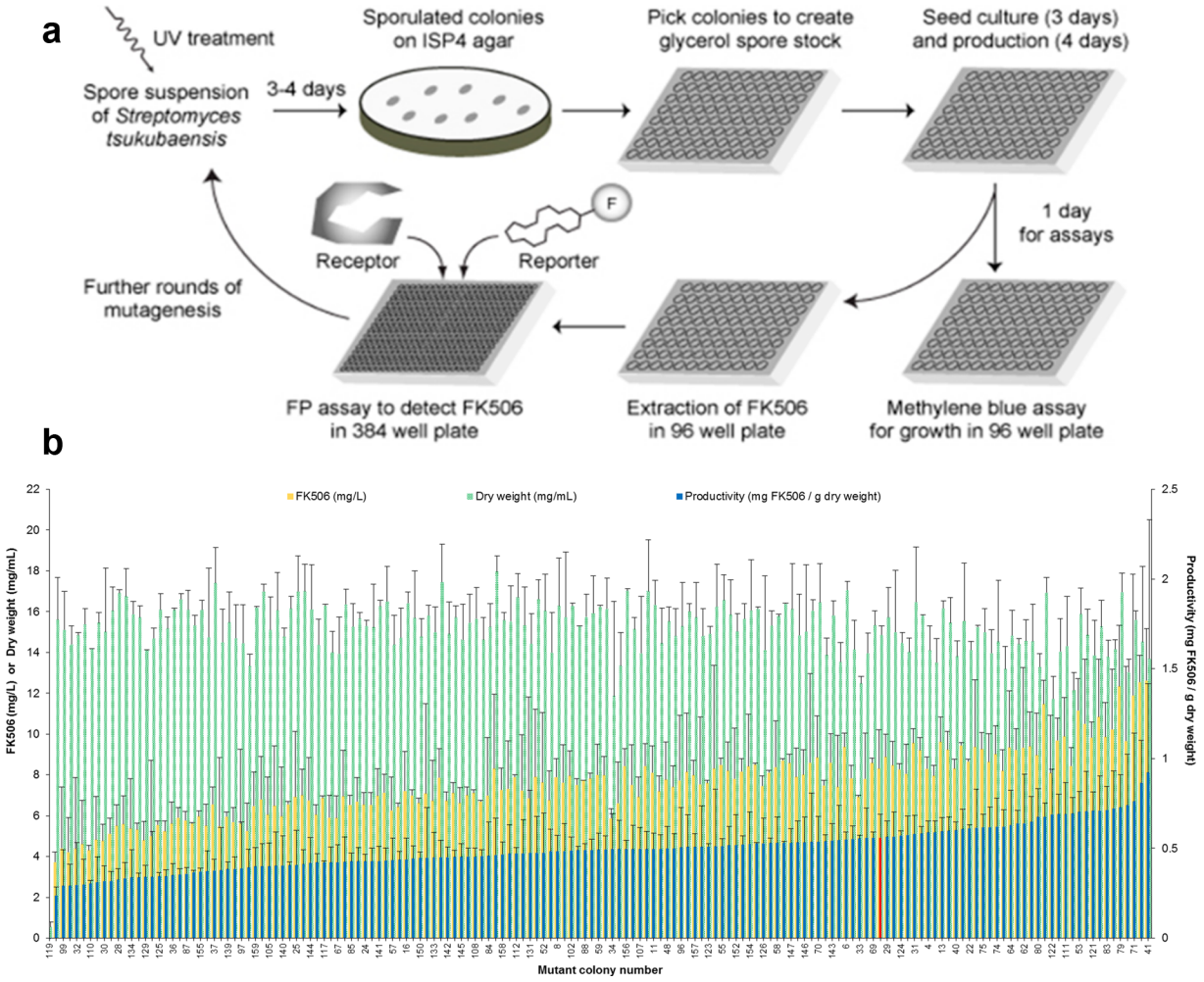

Figure 4. High-throughput mutagenesis and screening platform. (a) Integrated protocol for mutagenizing and screening a library of spores for improved production. (b) Productivity of mutant colonies (bottom red bars, *y*-axis on the right) arranged in ascending order from left to right. Only half the colonies are labeled. The bright red bar represents the average of 16 wells of nonmutagenized (WT) spores. For all other mutant colonies, bars represent the average of two wells grown in different plates. The amount of FK506 extracted (middle gray bars, *y*-axis on the left) and dry weight of cells (upper green bars, *y*-axis on the left) are also shown. Error bars represent the standard deviation for the two replicates. All FK506 values were background (production medium alone) subtracted.

99% kill rate and plated on ISP4 agar. Sporulated colonies appeared after 3−4 days. These were picked to create a glycerol spore stock in a 96-well plate.[44] The glycerol stock plate was used to inoculate a seed culture plate (3 days), followed by production plates (4 days) to produce FK506. Overall, one round of mutagenesis, production, and screening took ∼12 days (Figure 4a), which was ∼3 times faster than in a similar study.[45] In that study, the production of rapamycin was detected using growth inhibition of reporter cells on agar plates, which required incubation for 2 days, not including the time required for measurements. In contrast, the FP assay requires only 2 h of incubation time and 10^4−10^5 samples can be measured per day by a single plate reader.

The mutant colonies displayed productivities that range from 47 to 160% of the nonmutagenized spores (Figure 4b and Table S3). In general, the larger the amount of FK506 produced (milligrams per liter of medium), the higher the productivity of the cells (milligrams of FK506 per gram of dry weight), because cell growth (dry weight per milliliter of medium) overall does not change with FK506 production (Figure 4b). However, in some instances such as colony 43, the higher productivity value despite a smaller amount of FK506 can be explained by a lower rate of cell growth. Also, colony 119 was not included in the analysis as the cells did not grow in the production plate (Table S3). This may be due to experimental error or UV-induced mutations that impaired the growth of the cells. Thus, during the selection of mutants for scaled-up testing or further rounds of mutagenesis, both the productivity value and cell growth/density have to be taken into account. Cell growth/density is an important parameter in biosynthesis because it is related to the robustness of the cells, culture duration, and efficiency of feedstock to product conversion.

Next, to verify the top mutants identified by the FP assay, we scaled up production of the top four mutants (by productivity of FK506) in shake flasks. Production in flasks was performed by inoculating 4.5 mL of BaSa with 500 μL of spores and incubating the mixture for 40 h at 28 °C and 230 rpm. The seed culture was used to inoculate 5 mL of ISPz production medium (to a final OD_{600} of 1) in 50 mL unbaffled flasks (Pyrex Erlenmeyer 50 mL, CE-FLAS050) and incubated for 8 days at 28 °C and 230 rpm. The results show that on average, the four mutants produced levels of FK506 per gram of cells higher than that of the nonmutagenized strain (WT) (Figure

DOI: 10.1021/acs.biochem.7b00602
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5). However, the production yields of all the strains in shake flasks were highly variable. This was despite repeating the flask

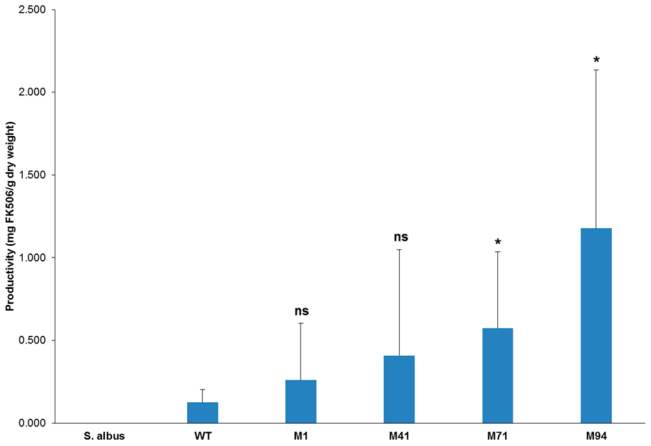

Figure 5. Productivity of the top four mutants cultured in shake flasks. *Streptomyces albus* was cultured as a negative control as it is not expected to produce FK506. WT represents the nonmutagenized strain. All bars represent the mean of nine replicates (three separate trials using three flasks per mutant), and error bars represent one standard deviation. All FK506 values were background (production medium alone) subtracted. Notations: ns, not significant ($p > 0.05$); *, significant ($p \leq 0.05$), compared to WT by a two-sample t test.

production trials three times, each time using three flasks per strain (total of nine replicates per strain). The high variability in shake flask cultivation compared to multiwell plate cultivation has also been previously observed.[47] Thus, for the purpose of library screening, it may be better to cultivate the strains first in multiwell plates for testing, before scaling up to shake flasks or bioreactors.

The performance of the FP assay was evaluated using the Z' score. The Z' score is a statistical parameter that takes into account the assay dynamic range and the variability associated with measurements and, thus, reports on the suitability of the assay for high-throughput screening applications.[48] We used the FP assay to screen a total of 96 wells of *Streptomyces albus* (non-FK506 producer) and 96 wells of M71 (one of the top mutant producers of FK506). The Z' score[48] was calculated using the equation $Z' = 1 - (3\sigma_n + 3\sigma_p)/(\mu_n - \mu_p)$, where σ_n is the standard deviation of the negative control (*S. albus*, a nonproducer), σ_p is the standard deviation of the positive control (M71, a mutant exhibiting an increased rate of FK506 production), μ_n is the mean FP value for *S. albus*, and μ_p is the mean FP value for M71. The Z' score was determined to be 0.81 (Figure 6), which falls within the classification of an excellent assay (an ideal assay has a Z' score of 1).[48]

We expect that the FP assay can be quickly and easily adapted to screen for a wide range of small molecule targets, because the FP assay decouples the target binding event from signal generation. This is different from recent assays that rely on transcription factors[16] or riboswitches,[15] in which the receptors undergo coupled target binding and signal generation events. For a new target, it is arguably easier to generate a pure binder than to create an allosteric receptor. In addition, unlike assays based on fluorophore quenching in the binding site[49] or bioluminescence resonance energy transfer (BRET),[50] in which the receptors are modified with a quencher or fluorophore, respectively, the FP receptor does not need to be modified.

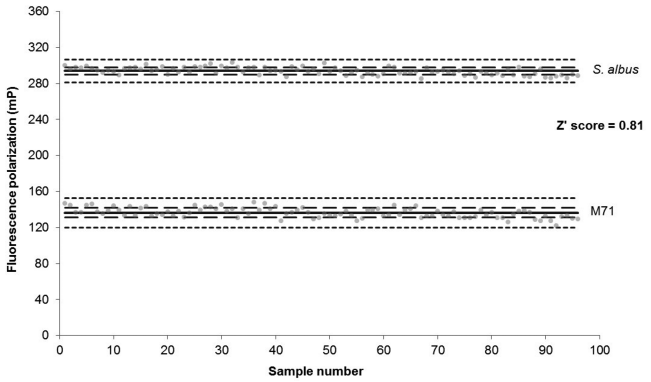

Figure 6. Z' score for the FP assay. Faint dots represent FP values for the 96 samples of *S. albus* (nonproducer of FK506) and 96 wells of M71 (one of the top mutant producers of FK506). The figure shows the mean of the control (—), one standard deviation from the mean (– – –), and three standard deviations from the mean (---).

Thus, to establish an FP assay for a new target molecule, two components are needed: a receptor and a reporter molecule.

We expect that for many small molecule targets, protein-based receptors can be found in nature, including its natural receptor or the inactivated enzyme for which the molecule is the substrate or product. Ideally, the receptor should bind tightly to the reporter and target molecules (K_d in the nanomolar to low micromolar range), because weak affinities require high receptor concentrations for the observation of a change in the FP signal, which could lead to protein aggregation or nonspecific binding with the reporter molecule or fluorophore. A receptor can also be created *de novo* by computational design and have its binding affinity increased via established directed evolution technologies (e.g., phage, mRNA, or yeast surface display).[29] The receptor can also be an antibody/monobody, RNA-based molecule (e.g., aptamers) or other binder.[51] The specificity of the FP assay rests on the specificity of the receptor. In this study, receptor FKBP12 was used. Because several ligands bind to FKBP12 with high affinity, including FK506, rapamycin, and other analogues, this assay can be adapted to detect any of those targets. Thus, it is important to verify the identity of the product using an independent method such as mass spectrometry. If a higher degree of specificity is desired, the receptor can be altered using directed evolution to selectively bind to one target molecule over other analogues.

The reporter molecule consists of a receptor-binding moiety and a fluorophore. It can be made by coupling the target molecule (or other analogues/inhibitors) to a fluorophore via a chemical linker. However, a potential limitation is that the sterically large fluorophore may decrease the affinity of the reporter for its receptor. This can be mitigated by coupling the fluorophore to sites on the target/inhibitor that are not crucial for receptor binding, as informed by X-ray crystal structures of the target/inhibitor–receptor complex or structure–activity studies.[52,53] Also, the linker length should be optimized. It should not be too long because that would allow the fluorophore to rotate even when it is receptor-bound (the "propeller effect").[54] Another important factor is the choice of fluorophore. If it interferes with receptor binding, smaller fluorophores can be tested. It is also important to verify that the reporter is bound specifically to the receptor (and does not interact nonspecifically via the fluorophore), by displacing it with a high concentration of the target molecule. Also, the

DOI: 10.1021/acs.biochem.7b00602
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quantum yield of the fluorophore influences signal intensity (and thus assay sensitivity).

Because the FP signal depends on the ratio of bound to unbound reporter molecules, the assay is not affected by light absorbance or color quenching by other compounds in the medium. Also, unlike competition assays using radioactive ligands,[55] the FP assay is homogeneous; there is no need for tedious washing steps to separate the bound and unbound reporter molecules. Furthermore, because the FP assay relies on a fluorescence signal, the assay is highly sensitive and can be performed in microtiter plate format. Measurements can be performed by a plate reader that contains a FP module. In this study, 384-well plates were used for the FP assay, which would potentially allow for screens of up to 10^4 samples per day (~26 plates, 30 min per plate). The FP assay can also be performed in 1536-well plates, which would increase sample throughput to ~10^5 samples per day.[56,57] This makes the FP assay cheap, automatable, and high-throughput compared to GC/LC−MS.

We have established that the FP assay can be used for small molecule detection. As a proof of concept, we adapted the FP assay for high-throughput screening of FK506 produced by *S. tsukubaensis* cultivated in 96-well microtiter plates. A FK506 production and extraction protocol in 96-well plates was developed to enable a high-throughput plate production and FP screening cycle. We applied our platform to screen a UV-mutagenized library for improved production strains. We further verified the top four mutants in shake flasks and showed that the FP assay is robust using the Z' score. Overall, the platform developed here will be useful not only for screening mutagenized strain libraries but also for other potential applications, including screening different culture conditions and medium compositions to optimize the production process. We expect that the FP assay will be broadly applicable to screening for other targets of interest to the metabolic engineering community and beyond.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00602.

Tables S1−S3 and additional references (PDF)

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Funding

Y.Z.N. was supported by a PhD fellowship from the Agency for Science, Technology and Research (A*STAR), Singapore. This work was supported by National Institutes of Health Grant R01 GM096064.

Notes

The authors declare the following competing financial interest(s): Y.Z.N., P.B.A., and V.W.C. are inventors on a patent filed regarding this method.

■ ACKNOWLEDGMENTS

The authors thank the Stockwell lab for access to the Victor X5 plate reader, Anna Kaplan and Rea Globus for help with initial FP trial experiments, Jimin Park for providing the *S. albus* strain, and Ehud Herbst for critical reading of the manuscript.

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