A Method for Fast, High-Precision Characterization of Synthetic Biology Devices

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Abstract

Engineering biological systems with predictable behavior is a foundational goal of synthetic biology. To accomplish this, it is important to accurately characterize the behavior of biological devices. Unfortunately, prior characterization efforts have not acquired sufficiently high-resolution data to enable predictive design. However, in the TASBE project we have developed a new characterization technique capable of producing such data. This document discusses the techniques we have developed, along with examples of their application.

Do we need to define what TASBE stands for?

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1 Motivation and Overview

DNA part characterization has foundational significance in the field of synthetic biology. From the inception of synthetic biology, the vision has been one of standardized parts that allow for predictable design, such as basic parts in electronics (e.g., TTL part data sheets). However, there have been two great obstacles in realizing this vision:

1. obtaining accurate measurements of relevant chemical properties within individual cells, and
2. predicting the behavior of a single DNA component with certainty when used in a novel, compositional design.

The second obstacle is predicated on our ability to make progress on the first; it is not possible to predict what behaviors should be measured from a novel system if it is not possible to obtain accurate measurements of the reference system needed to make the prediction. During the TASBE project, we had difficulty acquiring high-precision characterization data. Without sufficient part characterization, the ability to use parts in different contexts is significantly diminished, causing each additional use of an existing part to involve long, difficult, and costly debugging and experimentation.

The TASBE project's tight coupling between wet-lab work and high-level design tools has provided us with a clear set of requirements for DNA part characterization. This document describes the TASBE characterization process (Figure 1) that enables the construction of a library of biological computing devices with input/output relation characterization sufficient to enable predictive design. The remainder of this section describes the requirements for successful DNA part characterization (Section 1.1) and a comparison to prior methods (Section 1.2). Section 2 describes the TASBE characterization method. A set of best practices are described in Section 3, and an example is provided in Section 4.

1.1 Requirements for DNA Part Characterization

Our work in TASBE has shown us that, with regards to DNA part characterization, any type of predictive design will need at least:

- Large numbers of single-cell measurements (as opposed to population average values),
- Measurements of the level of part output signal(s) across the full dynamic range of levels of part input signal(s),

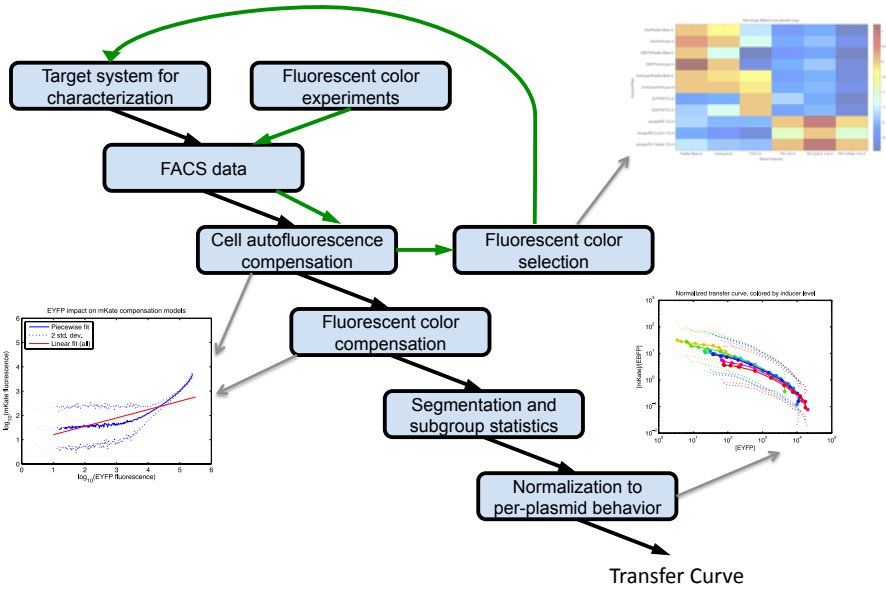


Figure 1: A visual summary of the steps in the TASBE characterization process. Details of these steps can be found in Section 2.

- Data to determine the per-copy effect of the construct, and
- The statistical distribution of single-cell output levels for each input level, in order to estimate the variability of behavior.

The reason for these requirements is that much of the interaction between parts takes place within individual cells, rather than between cells, across the whole population (except for special case systems involving intracellular communication). Thus, we measure the behavior of single cells. Cells exhibit a high degree of behavioral variability within a population, so to control for this we acquire a large number of single-cell measurements.

Finally, when evaluating DNA parts in a digital logic context, it is typically required for parts to have an approximately sigmoidal response with well-defined high and low input and output signal levels. Each DNA part has different output signal levels and make high-to-low transitions at different input signal levels. Thus predictable composition requires knowledge of the full input/output relation, particularly when working with parts where the transition between low and high expression has a slope that is not very steep.

Additionally we evaluate the per-copy (per-plasmid) behavior of the constructs in order to predict overall signal levels, since the number of copies of a system in a cell can vary greatly both by design (e.g., multiple copies, plasmids with different mean copy numbers) and through natural cell dynamics (e.g., copy number variation, transient transfection).

1.2 Comparison with Prior Techniques

Although others have recognized this same set of requirements, many prior efforts at characterization, not driven by a tight coupling with high-level design efforts, have gathered data that is insufficient for predictive design. For example, the variable strength library of promoters generated by the Collins lab [**?**] were only characterized for high and low expression level. The BioBricks spec sheet produced by Canton et al. [**?**] gathered data across the full dynamic range, but the transfer curve reports only population averages (the standard deviations shown are differences in population mean), as does the ongoing BIOFAB project [**?**]. Elowitz et al. [**?**] successfully predicted a circuit result, but the result was for an integrated, feedback circuit; it remains unclear how well the result generalizes. As far as we can tell, the data sheets produced by Imperial CSynBI [**?**] do not have the needed full-range transfer curves. Prior work by Weiss [**?**] does not calculate the parts on a per-copy basis. None of these previous efforts satisfied all four requirements above, and thus cannot produce the kind of characterization data that is necessary for predictable part composition.

2 Method

The TASBE characterization process is based on Fluorescence Activated Cell Sorting (FACS), a standard flow cytometry technique that allows large numbers of single-cell fluorescence measurements to be obtained quickly. These single cell measurements capture variation in device behavior, a necessity when composing devices within a cell.

The TASBE characterization process produces high-precision per-plasmid behavior data by adding three new elements:

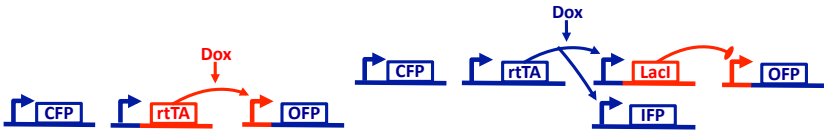
- A constitutive fluorescent protein that allows measurement of the number of copies of a system in the cell (introduced by the high variability in number of transfected or transformed plasmids),[1]
- Fluorescent protein/read channel screening to ensure $< 1\%$ bleed-over[2] between colors, and
- Multi-dimensional data segmentation that greatly increases the signal-to-noise ratio in systems with a variable plasmid count.

The aim of this section is to describe our characterization process in sufficient detail so that it may be implemented in any laboratory for any organism.

Should we mention other chassis in this paper if it's all Mammalian data?

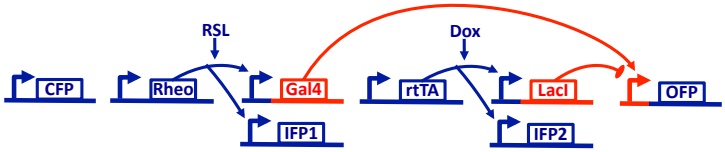
[1] Estimating plasmid count from constitutive fluorescent protein is not new, but using it as a part of characterization to obtain an input/output transfer curve is new.

[2] Because of spectral overlap of the read channels a color might be picked up in multiple channels. Bleed-over occurs when existence of one color is picked up on a channel that is not designated as the read channel of that color.



(a) Sensor Characterization

(b) Repressor Characterization



(c) Hybrid Promoter Characterization

Figure 2: A characterization system embeds the device to be characterized (red) with supporting constructs (blue) that control an input, measure input and output, and measure the number of copies of the system.

2.1 Constructs

Characterization of a device is done with a system composed of the following constructs:

- The device itself.
- A construct for externally inducing expression of each input to the device that is not directly controlled by experimental conditions (e.g., transcription factors).
- An *Input Fluorescent Protein* (IFP) for each input to the device that is not directly controllable. The IFP expression must be directly controlled by the same construct (e.g., promoter) that is controlling the input. This will be used to measure the input signal levels in each cell.
- An *Output Fluorescent Protein* (OFP) for each output of the device, whose expression is directly controlled by that output. This will be used to measure the output signal levels in each cell.
- A *Constitutive Fluorescent Protein* (CFP) that is expressed constitutively at a high level. This will be used to measure the number of copies of the system contained in each cell.

Figure 2 gives examples of how a characterization system can be instantiated, for a small-molecule sensor, repressor, and hybrid promoter.

In some organisms, it is possible to introduce multiple species of plasmid at closely correlated numbers (e.g., cotransfection via lipofection in mammalian cells). For such organisms, it is recommended that initially each functional unit

in the system be placed on its own plasmid, and the plasmids cotransfected or cotransformed. Thus, for example, a repressor characterization would be a 5-plasmid system (in Figure 2(b), CFP, rtTA, LacI, IFP, and OFP would be on separate plasmids).

A key advantage of separation onto multiple plasmids is eliminating the problems that may arise from larger constructs, such as interaction between adjacent functional units or low transfection yield. Note that if the plasmids can reproduce in the cell line (e.g., bacterial cells), it is important for them to have different origins of replication so that their relative copy counts do not drift.

2.2 Fluorescent Protein Selection

It is critically important to have extremely low spectral overlap when performing characterization. The reason is that, for any characterization, there will be some conditions where some proteins are highly expressed and others will have very faint expression. When the spectral overlap between fluorescent proteins leads to more than about 1% bleed-over of signal from one channel to another, even well-calibrated fluorescence compensation is often inadequate to produce good quantitative data.

In selecting fluorescent proteins for IFP, OFP, and CFP, therefore, it is necessary to begin by screening for a low-bleed-over combination of proteins and laser/filter pairs ("channels") used for FACS measurements.

To achieve this goal we have outlined a seven step procedure below. We first construct a bleed-over matrix (Steps 1-6) and then pick the best set of colors (Step 7) using this matrix.

1. Select a set of candidate proteins and a set of candidate channels for each protein.

2. For each protein under consideration, create a plasmid with only high constitutive expression of that protein.

3. Culture one set of cells for each protein, transfected/transformed with only the plasmid for that protein, along with a negative control (for computing autofluorescence). Ideally the negative control should be cells that have been transfected with a mock (non-expressing) plasmid, but cells that have not been transfected may be an acceptable substitute.

4. When cells are strongly expressing fluorescence, measure fluorescence via FACS on all channels under consideration.

5. For each channel c_i, compute autofluorescence mean $a_{\mu,i}$ and standard deviation $a_{\sigma,i}$. These should be arithmetic, since noise here is expected to be dominated by additive sensor noise.

6. For each protein / channel combination (p, c_i), compute the bleed-over b_{ij} with each other channel c_j as follows:

(a) Let m_x be the measured fluorescence on channel c_x.

(b) Select only those cells with an m_i far from both autofluorescence and saturation (e.g., in the $10^{3.5}$ to $10^{4.5}$ range).

(c) The estimated bleed-over b_{ij} is the arithmetic mean m_j/m_i for this subset of cells.

7. Select a set of protein / channel combinations (p, c_i) such that:

   - Mean fluorescence of p on c_i is at least two decades above $a_{\mu,i}+2\cdot a_{\sigma,i}$, and
   - $b_{ij} < 0.01$ for all other channels in the set.

Following this procedure should allow for the selection of a set of IFP, OFP, and CFP proteins and FACS settings that will provide for good quantitative characterization data to be gathered even when some expression is near autofluorescence levels. In mammalian cells, we have found the following combination to be effective:

- EBFP2, measured with a 405 nm laser and a 450/50 filter,
- EYFP, measured with a 488 nm laser and a 530/30 filter, and
- mKate, measured with a 561 nm laser and a 610/20 filter,

with iRFP likely to provide a good fourth.

2.3 Experimental Protocols

Experimental protocol will mainly be determined by the cell strain being used as the chassis for the device being characterized. Moreover, there is not yet a "standard" set of conditions under which devices must be characterized. Below is our basic protocol framework:

1. Introduce characterization system plasmid or plasmids into competent cells. If using multiple species of plasmid, use a protocol that results in correlated plasmid numbers (e.g., lipofection, but not nucleofection).
2. Culture cells in your preferred conditions.
3. Simultaneously, culture a control for the unmodified strain and a control for each fluorescent protein (e.g., a 3 color characterization system has a total of 4 controls) under the same conditions. The fluorescent protein controls should contain nothing but a constitutive expression of that protein, using the same promoter used for CFP in the characterization system.
4. To measure expression dynamics:

   (a) Select an evenly distributed set of sampling times, based on expected expression dynamics, as specified below in Best Practices (Section 3).

(b) Cultivate six colonies of cells for each time at which you plan to sample: three at high induction, three without induction. Cells should not be reused after FACS measurement.

(c) Acquire FACS data from three sets of high-induction cells and three sets of non-induced cells at each sampling time.

5. To measure an input/output transfer curve:

   (a) Select a set of induction levels which are somewhat uniformly distributed on the log-scale, as specified below in Best Practices (Section 3).

   (b) Cultivate three colonies of cells at each induction level, changing media as necessary to ensure cells are kept healthy and consistently induced.

   (c) Take FACS data when OFP is expected to near its maximum or minimum, whichever takes longer, as determined by expression dynamics experiments.

FACS data should be taken uncompensated: FACS software typically does not do a good job handling multi-laser compensation or compensation for autofluorescence, so compensation will be performed afterwards during analysis. FACS data should, however, be filtered to exclude events likely from debris rather than cells, but should not be filtered to exclude non-functional cells (that will be done later). Also run fluorescent beads for calibration, e.g., Spherotek rainbow calibration particles, to ensure that your FACS units can be converted to MEFLs.

update MEFL info as appropriate

Based on our own experiences, we recommend the following for mammalian cells from the HEK 293 FT cell line:

- Culture in DMEM medium (CellGro), supplemented with 10% FBS (PAA Laboratories), 2mM L-Glutamine (CellGro), 100x Strep/pen (CellGro), 100x Non-Essential amino acids (NEAA) (HyClone), and 10,000x Fungin (Invivogen).
- Passage cells with 0.05% Trypsin.
- Cotransfect plasmids via lipofection.
- Take FACS data at 72 hours, after resuspending cells in the appropriate media, such as 1xPBS that does not contain calcium or magnesium. Optionally select for plasmid using a resistance marker (e.g., with 2ug/ml of puromycin (Invivogen) for 2-4 days or until control cells that did not contain puromycin resistance are dead.)

2.4 Analysis

Once FACS data has been acquired, it can be analyzed in order to extract per-plasmid transfer curves relating device input level to output expression level. These can then be further processed as desired to extract other properties, such as Hill equation models or chemical kinetics.

There are four stages to data analysis:

1. Compensate for autofluorescence and spectral overlap.
2. Segment data into *bins* by both induction and CFP.
3. Compute statistics of each data points in each bin.
4. Normalize by CFP to obtain per-plasmid behavior.

2.4.1 Compensation for Autofluorescence and Spectral Overlap

The first stage of data processing is to map observed fluorescence levels to MEFLs. This is a three-stage process for each sample:

- Subtract mean autofluorescence $a_{\mu,i}$ from each channel's measurement m_i.
- Each measurement consists of $m_i = f_i + \sum_{j \neq i} b_{ji} f_j$, where f_i is the fluorescence from the protein that channel i is intended to measure and b_{ji} is the bleed-over from channel j to channel i. Solve simultaneous equations to obtain the set of f_i.
- Translate f_i from relative fluorescence to MEFLs using standard FACS bead-based calibration.

Note that many compensation methods (including those typically built into FACS machines) will not perform complete compensation of this sort, typically either compensating only within a particular laser or omitting autofluorescence compensation. It is also not safe to assume that a fluorescent protein will emit only on adjacent channels (many have surprising outlying lobes), or that a protein will emit precisely how the manufacturer's spectrum indicates that it will emit (as the cellular context may be sufficiently different). These distortions may have a major effect on your measured values, particularly for low fluorescence values.

2.4.2 Segmentation into Bins

The next step is to segment data into bins, simultaneously by induction conditions and logarithmic CFP intervals, and to throw away likely invalid data:

- Discard all samples with CFP below $2 \cdot \sigma$, where σ is the standard deviation of autofluorescence on the CFP channel. These are expected to be cells that failed to receive plasmids.

- Discard all samples with any fluorescence measure below 0, as they will cause problems with geometric statistics later.[3]
- Discard all samples with CFP fluorescence above f_{max}, set to be less than the expected saturation point for the FACS detectors.
- For a logarithmic partition, $\pi_C = \{[a, b]\}$, place all samples with $a < CFP \leq b$ into bin $[a, b]$. Suggested bin width is $log_{10}b/a \leq 0.25$.
- Discard all bins with less than a minimum m samples (suggested $m = 100$), as being statistically invalid.

Bins are logarithmic because expression noise is expected to be mainly multiplicative rather than additive. Binning is used because of the expected cell-to-cell variation in number of copies of the system; the differences can be caused by natural variation in copy number or transient transfection. As noted above, good characterization data requires knowing the per-plasmid behavior of the system. The observed behavior depends on the number of copies of the interacting parts (Figure 3), because the concentrations of chemicals produced from different copies add together. Consider the typical case of a single promoter/regulator interaction: when there are more copies of the promoter and the gene it regulates (a fluorescent protein in our case), then the same concentration of regulator will produce more fluorescence. Likewise, when there are more copies of the regulator gene, it takes less inducer to produce the same concentration of regulatory protein. What this means is that cells with different numbers of copies of a circuit may have radically different behaviors under the same conditions. An example of where this effect shows up strongly is lipofection of mammalian cells, where the number of copies of the plasmid that enter each cell typically range over two to three orders of magnitude.

Due to this variation in observed behavior, it is important to segment data not just by induction condition, but also by number of copies of the system, as indicated by CFP. The relation between observed CFP expression and number of copies is not, however, linear, as will be explained in detail below in Section 2.4.4.

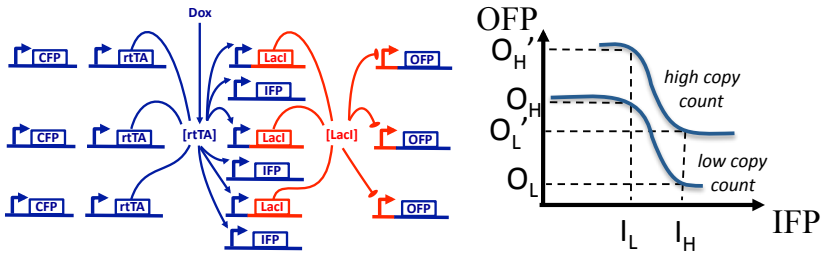
2.4.3 Computation of Bin Statistics

Within each bin, compute the **geometric** mean and standard deviation for each fluorescent value for all data points within that bin. Geometric mean and standard deviation can be taken by computing the ordinary mean and standard deviation on a log scale:

$$\mu_g(x_i) = e^{\mu(lnx_i)}$$
$$\sigma_g(x_i) = e^{\sigma(lnx_i)}$$

It is important to use geometric rather than arithmetic statistics because expression noise on the fluorescent proteins is typically multiplicative rather

[3]This will cause upward distortion in measurements near the autofluorescence floor, and should be improved in the future by better joint estimation methods.



(a) (b)

Figure 3: When there are multiple copies of a characterization system, the chemical concentrations superpose (a). This affects the observed device input and output behavior differently (b). For the input, more copies means that less inducer is needed to get the same input concentration. On the output, the reponse to a given input concentration is multiplied by the number of copies.

than additive. Using arithmetic means will result in statistics that are skewed upwards by high outliers.

Remember that to compute error ranges, the geometric mean is multiplied and divided by the geometric standard deviation, not added and subtracted. For example, a range of two standard deviations is:

$$[\mu_g/\sigma_g^2, \mu_g\sigma_g^2]$$

2.4.4 Normalization to Obtain Per-Plasmid Behavior

Transforming bin statistics into per-plasmid behavior takes two steps:

- Estimate mean number of plasmids from mean CFP.
- Divide mean OFP by estimated mean plasmids.

Note that the standard deviations and the IFP mean are *not* transformed. The standard deviations are not transformed because they are multiplicative and not additive; an increased number of plasmids may actually tighten the observed standard deviation. The IFP mean is not transformed because the devices respond to the chemical concentration created by the combined production of all inputs, not the per-plasmid production of inputs.

Estimating Plasmid Count The expression of MEFLs from a single plasmid can be determined in a number of different ways. For example, in mammalian cells with transient transfection, plasmids do not replicate and so, as the cells divide, the number of plasmids will decrease, until eventually all cells have either

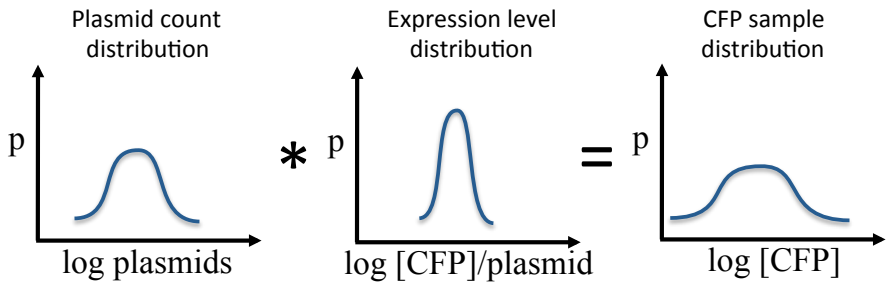


Figure 4: The distribution of observed CFP samples (CFP) is the convolution of the distribution of plasmid count (L) by the distribution of expression levels (V). Since these are typically both gaussian, it means that samples in any given bin are biased toward the mean level of CFP.

one or zero plasmids, at which point expression can be measured.[4,5] Let the mean expression for a reference constitutive promoter (e.g., the CAG promoter in mammalian cells) from a single plasmid be called E.

Estimating the number of plasmids from the CFP in a bin will use this measure, but also must compensate for sampling bias caused by the underlying plasmid count distribution. A naive estimate of the mean number of plasmids in a bin can be taken simply by taking the mean CFP from samples in the bin and dividing by E MEFLs/plasmid. This gives a reasonable estimate for bins near the mean overall CFP level, but as bins move away from the center, the estimate will be more and more incorrect.

The reason for this is that the distribution of CFP is the *convolution* of the distribution of plasmid count and the distribution of expression levels (Figure 4), and expression noise is typically larger than the range of a bin. When both of these distributions are gaussian in shape (as is often the case) and the variation in expression levels is relatively independent for each fluorescent protein, this means that in any given bin the samples are biased toward cells closer to the mean number of plasmids, simply because there are so many more of those cells. This is illustrated in Figure 5.

Next we will discuss the procedure that will correct the sampling bias effect. We use the notation:

μ the geometric mean of a distribution

σ^2 the geometric variance of a distribution (σ is the standard deviation of the distribution)

Going back to Figure 4 we need the mean and variance for the three distributions, L : plasmid counts, V: Expression level and CFP: Observed CFP. Some

[4]Typically we do not wait for this point to collect data, but in order to determine the expression from a single plasmid we need to collect data when the cells only have one plasmid.

[5]Assuming appropriate degradation tags or computational models are used to cancel out the inherited levels.

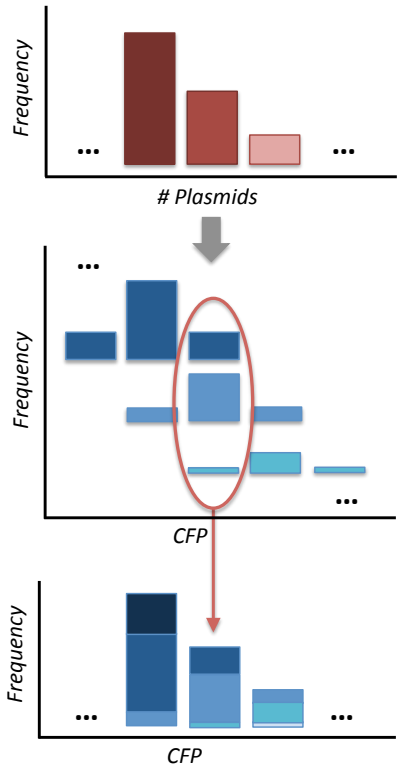


Figure 5: The frequency of different plasmid counts varies (top). The expression of CFP from these plasmid counts is noisy and larger than the bin range (middle). Thus the observed frequencies of CFP levels are biased toward cells closer to the mean number of plasmids.

of these statistics are computed from the data and others need to be estimated:

- The mean and variance of CFP, μ_{CFP} and σ^2_{CFP}, are directly computed from the data
- The mean expression level, μ_V, is E and,
- We can estimate the variance of expression level, σ^2_V, by computing the observed (non-CFP) expression variance in each bin.
- The mean plasmid count is estimated as: $\mu_L = \mu_{CFP}/\mu_V$ (using convolusion theorem)
- The variance of plasmid count is estimated as: $\sigma_L = \left|\sqrt{\sigma^2_{CFP} - \sigma^2_V}\right|$

Things to fix: 1) Geometric variance for convolution 2) $p(CFP|L)$ is undefined

Once we have the three distributions, L, V and CFP, we can apply the Bayes Theorem to compute the expected plasmid count in a cell given an observed CFP level. We choose to operate on the discrete level for these computations thus we partition the space of plasmid counts and observed CFP levels into bins. Let π_L be a partition of the space of plasmid counts into bins $[x, y]$. Then

- Probability of a cell containing plasmids in the range $[x, y]$ given that it has CFP levels in the range $[a, b]$ is estimated by:

$$p(L \in [x,y]|CFP \in [a,b]) = \frac{p(CFP \in [a,b]|L \in [x,y]) \cdot p(L \in [x,y])}{\sum_{[x,y]\in\pi_L} p(CFP \in [a,b]|L \in [x,y]) \cdot p(L \in [x,y])}$$

- Expected number of plasmids in a cell given that it has CFP levels in the range $[a, b]$ is:

$$E(L|CFP \in [a,b]) = \sum_{[x,y]\in\pi_L} \mu([x,y]) \cdot p(L \in [x,y]|CFP \in [a,b])$$

Details of the derivation for estimates For readers who are interested in the mathematical derivation of the estimates, the details are as follows: Given the distributions for V and CFP, we can calculate the underlying plasmid distribution (μ_L, σ_L) using the convolution theorem adapted for geometric mean and variance:

$$CFP = V * CFP$$
$$\mu_{CFP} = \mu_V * \mu_L$$
$$\mu_L = \mu_{CFP}/\mu_V$$
$$\sigma^2_{CFP} = \sigma^2_V + \sigma^2_L$$
$$\sigma_L = \left|\sqrt{\sigma^2_{CFP} - \sigma^2_V}\right|$$

Letting π_L be a partition of the space of plasmid counts into bins $[x, y]$, the distribution of plasmids contributing to each CFP bin $[a, b]$ can be estimated using Bayes' law:

$$p(L \in [x,y]||CFP \in [a,b]) = \frac{p(CFP \in [a,b]|L \in [x,y]) \cdot p(L \in [x,y])}{p(CFP \in [a,b])}$$

$$p(L \in [x,y]|CFP \in [a,b]) = \frac{p(CFP \in [a,b]|L \in [x,y]) \cdot p(L \in [x,y])}{\sum_{[x,y]\in\pi_L} p(CFP \in [a,b]|L \in [x,y]) \cdot p(L \in [x,y])}$$

where the $p(CFP \in [a, b]|L \in [x, y])$ term is taken from the observed expression variation model and the $p(L \in [x, y])$ term is taken from the plasmid distribution model.

Finally, the mapping from mean CFP level to mean plasmid count can be produced by taking the expectation of plasmid counts:

$$E(L|CFP \in [a,b]) = \sum_{[x,y]\in\pi_L} \mu([x,y]) \cdot p(L \in [x,y]|CFP \in [a,b])$$

2.5 TASBE Analysis Package

The TASBE Analysis Package is a collection of software intended to make using this characterization method simple, by providing implementations of all of the necessary data processing. It consists of a collection of Matlab functions for fluorescent protein screening, construction of fluorescent compensation models, and analysis of characterization data.

The package can be downloaded from `http://synthetic-biology.bbn.com/`, and is available under a permissive free software license.[6] It includes a README and example files that show how to use the package to process characterization data. See also Section 4 below.

Actually put this up on the web :-)

3 Best Practices

For ensuring that results can be useful and trustworthy, we recommend the following best practices:

- All constructs other than the device being characterized should be in the same direction on the same strand of DNA.
- Fluorescent proteins should have $< 1\%$ bleed-over.
- Controls should include fluorescent beads for FACS calibration, the strain with a mock (non-expressing) transfection (for autofluorescence), and constitutive expression of each fluorescent protein—one control per protein (for fluorescence calibration). An unmodified strain may be an acceptable alternative for measuring autofluorescence.
- Consistent experimental protocols, e.g., time, laser power, amount of DNA, should be used for all devices intended to be interconnected.
- Induction levels should be evenly logarithmically distributed, with at least three induction levels per decade, across at least three decades (e.g., 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1000 nM). There should also be a non-induced case (e.g., 0 nM), except for sensors where this is not possible (e.g., induction by temperature).
- Time sequence data for expression dynamics should be timed based on the time for approximately full expression of the constitutive protein (T_C). They should be evenly distributed, both before and after T_C, including at least ten samples, starting no later than $0.25T_C$ and ending no earlier than $3T_C$.
- Fluorescence values should be taken from at least 10,000 cells per induction level with a recommended cell count of 50,000 per induction level (after gating to remove likely non-cell FACS events).

[6] The license is "GPL with linking exception," meaning intuitively that you can use or build on top of the software however you like, but that if you improve the core software you need to contribute your improvements back to the community.

- Each bin should have a constitutive fluorescence range of at most a quarter decade.
- Each bin used should contain at least 100 data points.
- Results should present the per-cell mean and standard deviation of each bin. Mean and standard deviation should be geometric.
- Experiments should be conducted in triplicate. This should be used to compute the mean and variance of both the per-cell means, the per-cell standard deviations, and the per-experiment variation.
- Plasmid sequences should be verified, preferably by sequencing, though restriction digest is an acceptable substitute when the sequences of component DNA parts have previously been verified. Constructs should not be made using error prone processes such as PCR; recommended assembly processes use restriction cloning or homologous recombination. Examples of recommended processes include BioBricks and Gateway/Gibson.
- Experiment records should include at least: strain of cells, DNA sequence of plasmid constructs (preferably encoded with SBOL[7]), the protocol used for introducing plasmids to cells, culturing conditions, and number of hours cultured before measurement.

4 Usage Example

4.1 Fluorescent Protein Selection Example

As described in Section 2.2, a first critical step is to determine which fluorescent colors are good candidates to use in circuits on a given FACS machine. This example uses a BD LSR II FACS machine with the following laser/filter combinations ("channels"):

Pacific Blue-A 405 nm laser with 450/50 filter,

AmCyan-A 405 nm laser with 510/50 filter,

FITC-A 488 nm laser with 530/50 filter,

PE YG-A 561 nm laser with 575/26 filter,

PE-Cy5-5 YG-A 561 nm laser with 695/40 filter, and

PE-TxRed YG-A 561nm laser with 610/20 filter.

Cells cultures, each constitutively producing a different fluorescent protein of interest, are processed with the FACS machine. This example examines the following fluorescent proteins: Cerulean, EBFP2, AmCyan, EYFP, EGFP, and mKate.

[7] `http://www.sbolstandard.org/`

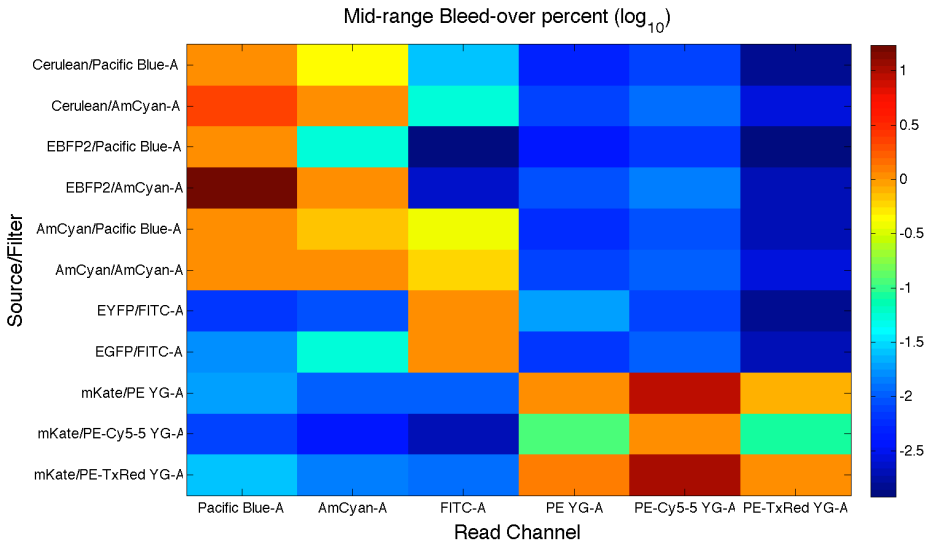


Figure 6: The heat map representing possible fluorescent proteins and their spectral overlap on the available read channels. Blue colors indicate less overlap while reds indicate significant overlap.

Figure 6 shows the resulting FACS data represented as a heat map. This can be used to identify good combinations of colors to use in characterization constructs. Each row of the heat map is a fluorescent protein and a target channel, for example the first row is the Cerulean fluorescent protein measured using the Pacific Blue-A channel. Each column in the heat map represents an available read channel on the FACS machine. As we want to minimize the spectral overlap between colors, the heat map plots the mid-range bleed-over percent between colors on a log_{10} scale. Further, as shown in Figure 7, the bleed-over for the target channel is 100% or 1, which in log_{10} is 0. Figure 8 highlights the EBFP2/AmCyan-A row. Here the AmCyan-A channel has a zero value. EBFP2 has high bleed-over on the Pacific Blue-A channel (dark red) but low bleed-over into the FITC-A, PE YG-A, and PE-TxRed YG-A channels (darker blues, $< 1\%$ bleed-over), and moderate bleed-over into the PE-Cy5-5 YG-A channel (lighter blue, $\sim 3\%$ bleed-over).

We are looking for three pairs of fluorescent proteins and channels that produce unique hot values on the read channels. An example of a bad combination of fluorescent colors is shown in Figure 9. In this case, Cerulean and EBFP2 both produce high readings on the Pacific Blue-A and AmCyan-A channels. It would be impossible to separate the measurements on these channels into Cerulean and EBFP2 values.

Figure 10 shows the fluorescent proteins and channels we selected: 1) EBFP2 with the Pacific Blue-A channel, 2) EYFP with the FITC-A channel, and 3) mKate with the PE-TxRed YG-A channel. While any of the PE YG-A, PE-

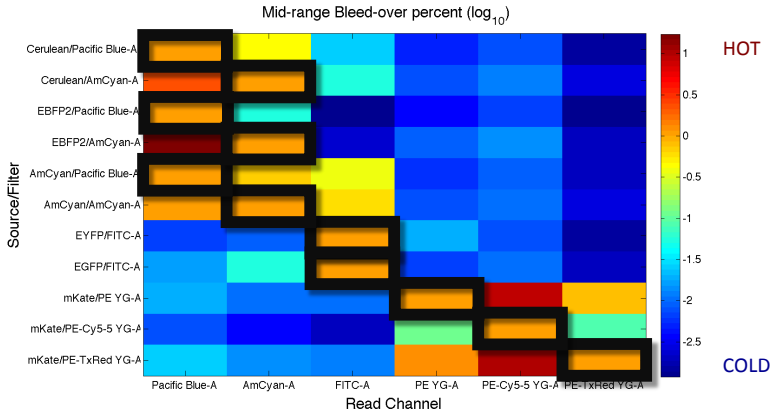


Figure 7: The values in each row are zero for the target channel (bleed-over is 100% or 1, and $log_{10}1 = 0$).

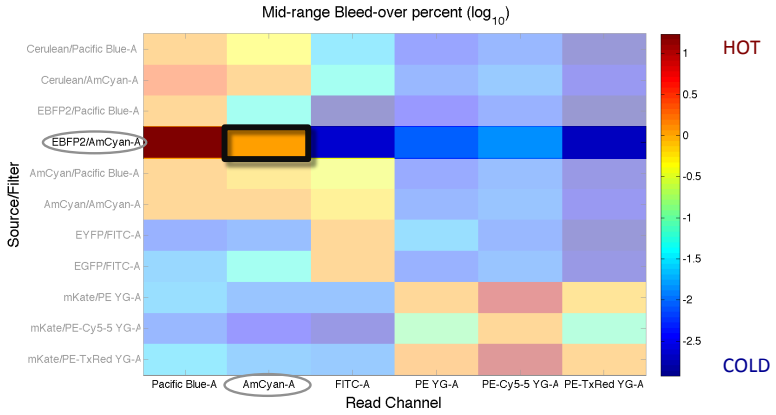


Figure 8: One possible source/channel combination is highlighted.

Cy5-5 YG-A, and PE-TxRed YG-A channels give high readings for mKate and low readings for EYFP and EBFP2, the selected channel gives a high reading for mKate and sufficiently low readings for the other colors. Our highest bleed-over is from mKate into the Pacific Blue-A channel (~2.3%) and FITC-A channel (~1.3%). All of the other bleed-overs are well under 1%.

4.2 Example Construct

Note that this example does not conform to all best practices – do what you can with the data you have. Figure 11 shows the circuit construct used for characterization for the LacI repressor. In this example, mKate is the CFP, EBFP2 is the IFP, and EYFP is the OFP. The fluorescent color levels are measured using

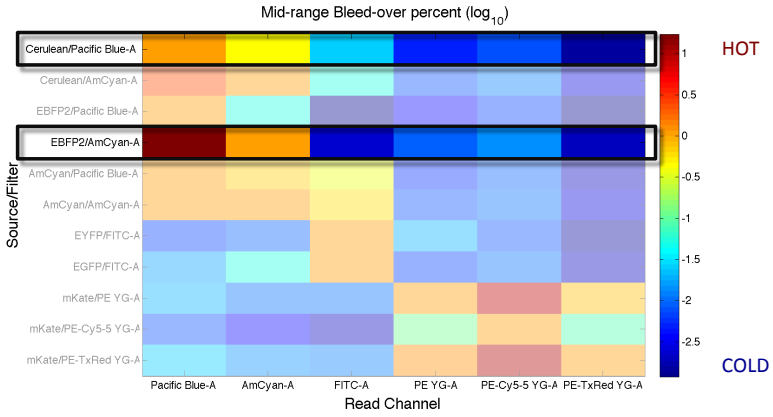


Figure 9: Two colors that would be a poor choice for use in the same characterization experiment. Cerulean and EBFP2 both have high readings on the Pacific Blue-A and AmCyan-A channels making them impossible to adequately separate.

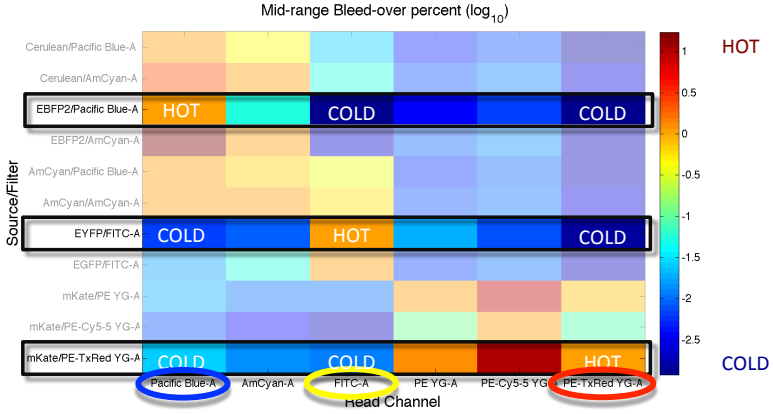


Figure 10: The fluorescent colors selected; the spectra of these colors do not overlap significantly making it possible to cleanly separate the data.

the "Pacific Blue-A," "FITC-A," and "PE-TxRed YG-A" channels.

4.3 Example Analysis

The TASBE Analysis Package (`http://synthetic-biology.bbn.com/`), contains an example analysis script called `analysis_example.m`. The example script first sets up the input, output, and constitutive colors and filters:

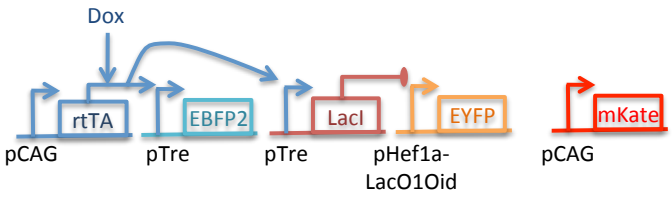


Figure 11: The circuit used to characterize LacI.

```
inputColor = create_color_index('blue','Pacific Blue-A','b');
outputColor = create_color_index('yellow','FITC-A','y');
constitutiveColor = create_color_index('red','PE-TxRed YG-A','r');
ioc = struct('input', inputColor, 'output', outputColor, ...
    'constitutive', constitutiveColor);
```

In addition to collecting data for cells containing the characterization construct, data should be collected for blank cells and cells constitutively expressing each of the fluorescent proteins. This best practice is important to verify that the FACS machine is collecting data correctly, and the data are used to calibrate the autofluorescence levels of the cells. Fluorescent beads should be used so that data can be converted to MEFLs. This example does not include conversion to MEFLs. The value of E (see Section 2.4.4) should also be determined. We have not yet conducted the experiment to determine E for this example and use 1 as a placeholder for this value. The color and blank data are specified in the Matlab file as follows:

```
blankfile= ...
    '../../2011-08-12-Single-LacI-Puro-72hr/08-12-11_blank_P3.fcs';
bluefile = ...
    '../../2011-07-29-Gal4_Tal1_color_comp/07-29-11_EBFP2_P3.fcs';
yellowfile = ...
    '../../2011-07-29-Gal4_Tal1_color_comp/07-29-11_EYFP_P3.fcs';
redfile = ...
    '../../2011-07-29-Gal4_Tal1_color_comp/07-29-11_mkate_P3.fcs';
```

In this example, the construct is evaluated at the following Dox levels (nM): 0, 1, 2, 4, 6, 10, 12, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 1000, and 15,000. When entering values in Matlab, 0.1 is used in place of 0 so that it can be plotted on a log scale. This is encoded in Matlab as:

```
dox = [0.1, 1, 2, 4, 6, 10, 12, 15, 20, 25, 50, 75, 100, 200, 300, ...
    400, 500, 600, 700, 800, 1000, 15000];
```

The data files are then loaded with the following:

```
clear A; clear filenames;
stem = '';
```

```
A = strvcat('05-14-11_0dox_001_P3.fcs', ...
'05-14-11_1dox_002_P3.fcs', ...
'05-14-11_2dox_003_P3.fcs', ...
'05-14-11_4dox_004_P3.fcs', ...
'05-14-11_6dox_005_P3.fcs', ...
'05-14-11_10dox_006_P3.fcs', ...
'05-14-11_12dox_007_P3.fcs', ...
'05-14-11_15dox_008_P3.fcs', ...
'05-14-11_20dox_009_P3.fcs', ...
'05-14-11_25dox_010_P3.fcs', ...
'05-14-11_50dox_011_P3.fcs', ...
'05-14-11_75dox_012_P3.fcs', ...
'05-14-11_100dox_013_P3.fcs', ...
'05-14-11_200dox_014_P3.fcs', ...
'05-14-11_300dox_015_P3.fcs', ...
'05-14-11_400dox_016_P3.fcs', ...
'05-14-11_500dox_017_P3.fcs', ...
'05-14-11_600dox_018_P3.fcs', ...
'05-14-11_700dox_019_P3.fcs', ...
'05-14-11_800dox_020_P3.fcs', ...
'05-14-11_1000dox_021_P3.fcs', ...
'05-14-11_15000dox_022_P3.fcs');
for i=1:size(A,1)
    filenames(i,:) = sprintf('%s%s',stem,A(i,:));
end;
```

A decision must be made about the bin range and width. This is done by examining the plot showing the count of cells per bin. At least 100 cells are required per bin. In this example the bin width was chosen to be $10^{0.25}$ and the bins run from $10^{1.5}$ to $10^{4.5}$. Note that our current script assigns bins before adjusting for the various fluorescent colors, so the bin values in Figure 12 are shifted (and some bins with less than 100 cells are used, contrary to best practices).

The following lines of Matlab code set the bin parameters:

```
bin_min = 1.5;
bin_max = 4.5;
bin_increment = 0.25;
```

The `do_analysis.m` script then performs the following steps, as described in Section 2.4:

1. Compensate for autofluorescence and spectral overlap.
2. Segment data into bins by both induction and CFP.
3. Compute statistics of each data points in each bin.
4. Normalize by CFP mapped to plasmid count to obtain per-plasmid behavior.

Update all code to match actual example

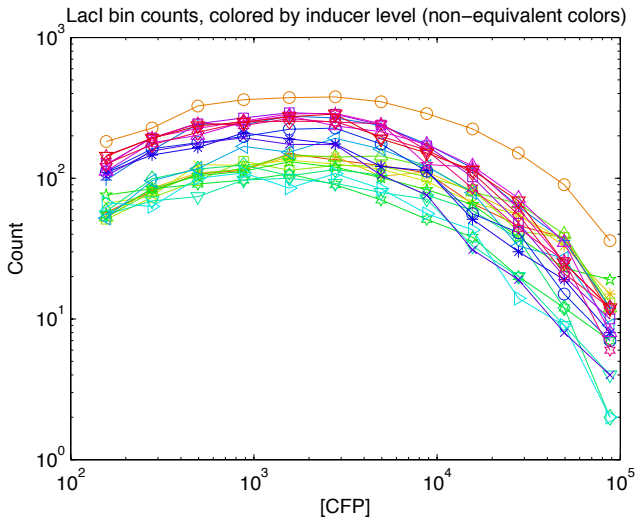


Figure 12: Bin counts from the LacI characterize data.

The following code specifies the file names to use for the graphs and data files and then runs the script:

```
population_prefix = 'LacInew';
inducer = 'DoxNew';
description = 'LacI−SplitCircuitNew';

do_analysis(ioc, blankfile, bluefile, yellowfile, redfile, dox, ...
    filenames, bin_min, bin_max, bin_increment, inducer, ...
    population_prefix, description);
```

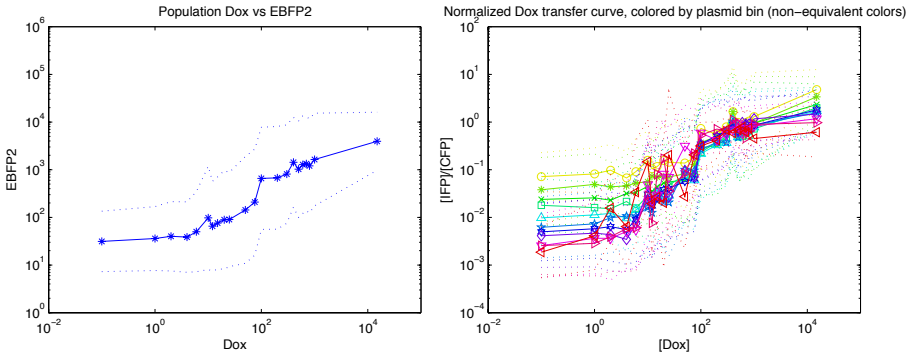
In future versions of this document we will explain the `do_analysis.m` script in detail.

Typical previous results would have examined the mean fluorescence of all the cells. First we examine the Dox transfer curve which would have produced the curve shown in Figure 13(a), but instead produces the curve in Figure 13(b). Notice the steeper curve and larger difference in the expression magnitude using the normalization technique. Because the input is externally controlled, though, the difference is not huge.

regenerate figures with newest code version

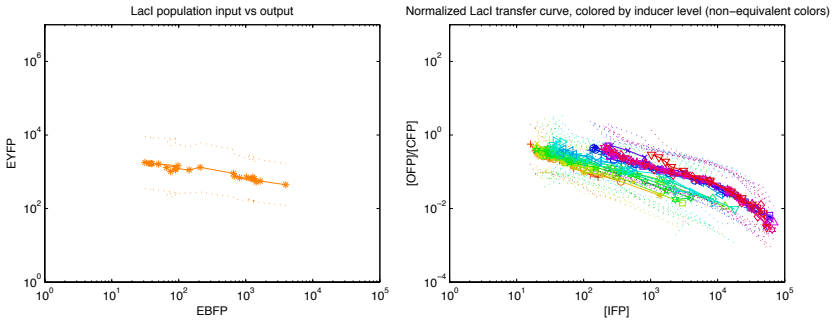
The difference in the LacI curve is more impressive. Without this technique there is about a 5x repression for LacI (Figure 14(a)). After applying our technique, about a 200xrepression is visible (Figure 14(b)).

revise number here and in caption



(a) Population (b) Normalized

Figure 13: Population fluorescence data for Dox (left) and normalized transfer curve colored by plasmid bin (right). Notice the increase the steeper curve and larger difference in the expression magnitude.



(a) Population (b) Normalized

Figure 14: LacI population fluorescence data (left) and normalized transfer curve colored by inducer level (right). The population curve has 5x repression while the normalized curve has about 200x repression.