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Short Communication

Experimental design of systems involving multiple fluorescent protein reporters

Loveleena Bansal [a,1], Randall Nelson [a], Eric Yang [a], Arul Jayaraman [a], Juergen Hahn [b,c,*]

[a] Department of Chemical Engineering, Texas A&M University, College Station, TX 77843, United States
[b] Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, United States
[c] Department of Chemical & Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, United States

HIGHLIGHTS

- Developed mathematical guidelines to select fluorescent reporters for simultaneous use.
- Mixtures of reporters are made using fluorescent protein-expressing *E. coli* strains.
- Contribution of reporters to overall mixture intensity is calculated using linear unmixing.
- D-optimal design is used to select proteins to maximize the estimation accuracy.

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ABSTRACT

Fluorescent proteins have found widespread applications for analysis of biological systems as they can be used to track various events within living cells. Multiple fluorescent proteins are also simultaneously used to monitor different aspects of biological systems. However, extensive overlap in the emission spectra of the fluorescent proteins poses challenges in extracting the contribution of individual proteins to overall fluorescence intensity measurements. This work addresses this issue by deriving a computational formulation for extracting the contribution of fluorescence intensities of individual reporters to the overall measurements taken using a plate reader. Then, this formulation is used for deriving an experimental design criterion for choosing sets of fluorescent proteins such that the accuracy of the estimated contribution of different fluorescent proteins is maximized. The results are validated using two sets of experimental data involving different sets of fluorescent proteins. This work represents the first quantitative study that evaluates experimental design for selection of fluorescent proteins to use simultaneously for multiple-labeling applications.

1. Introduction

Fluorescent proteins are now regularly used to monitor a variety of aspects of biological systems. Some uses of fluorescent proteins involve, but are not limited to, monitoring the expression of genes, protein localization and protein–protein interactions (Lippincott-Schwartz and Patterson, 2003; Van Roessel and Brand, 2002). The main advantage that the use of fluorescent proteins has over other approaches is that the fluorescence can be monitored in real-time without destroying the sample, thereby allowing monitoring of the location where fluorescence can be observed, taking several measurements to increase measurement accuracy, or to monitor aspects of an experiment over time.

There are now a number of fluorescent proteins that are commercially available (Nowotschin et al., 2009; Shaner et al., 2005) which can be simultaneously used for profiling of biological systems. The main advantage of concurrently using multiple fluorescent reporters, with different emissions spectra, is that it is possible to simultaneously monitor different components of a system or their interactions. For instance, a number of researchers have used multiple fluorescent reporters and multispectral imaging techniques to monitor complex protein–protein interactions (Hu and Kerppola, 2003; Waadt et al., 2008), protein and cellular movements (Hiraoka et al., 2002; Hoffman, 2005), transcriptional regulation due to multiple promoters (Cox et al., 2010) and for understanding morphological developments in in vitro or in

* Corresponding author at: Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Center for Biotechnology and Interdisciplinary Studies, rm: 4213 110 8th Street, Troy, NY 12180, United States. Tel.: +1 518 276 2138; fax: +1 518 276 3035.
E-mail address: hahnj@rpi.edu (J. Hahn).
[1] Present Address: Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, United States.

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in vivo imaging (Chen et al., 2012; Hoffman, 2005; Nowotschin et al., 2009). In addition, labeling with multiple fluorescent markers has also been used for determining the compositions of different bacterial species in biofilms (Cowan et al., 2000; Ma and Bryers, 2010). However, the combined use of multiple fluorescent proteins is challenging due to the fact that the emission spectra of different proteins overlap and any measurement involves contributions from all the fluorescent proteins. Thus, extracting the contribution of individual types of fluorescent proteins from measurements becomes very difficult.

A number of "unmixing" algorithms have been discussed in the literature to resolve the overlapping emission spectra of fluorescent reporters in measurements from samples containing two or more reporters at the same time. If only two reporters are present in a sample, one can choose the reporters with minimal overlap in their emission or excitation spectra and use appropriate filter sets to separately measure the intensity of the reporters (Cowan et al., 2000; Shaner et al., 2005). In a large number of applications involving three or more fluorescent protein reporters, the spectral contributions of the individual reporters are distinguished using imaging spectroscopy aided by a mathematical linear unmixing formulation in which the fluorescence intensity of each pixel in an image is assumed to be a linear combination of the intensities of the individual reporters (Dickinson et al., 2001; Lansford et al., 2001; Zimmermann et al., 2003). This technique has been implemented to distinguish the spectra of up to seven different fluorescent dyes (Tsurui et al., 2000). Additionally, flow cytometry has widely been used for high-throughput analysis at the single cell level to measure a very large number of fluorescence signals by using up to 30–40 optical filters for excitation blocking, spectral separation and transmission of narrow bands (Grégori et al., 2012; Perfetto et al., 2004). The overlapping excitation and emission spectra of fluorescent reporters has entailed the use of intricate and expensive experimental setups for their spectral resolution and remains a major challenge in multicolor applications.

In this work, a mathematical approach is developed for selecting the fluorescent proteins to use together for multiple-labeling applications in order to be able to reconstruct the contribution of individual proteins to the overall measurements. Two main tasks for selection of the fluorescent reporters have been addressed in this work: (1) solution of an unmixing problem to determine the contribution of individual proteins to the overall intensity of a sample containing two or more proteins, and (2) making use of this formulation to perform experimental design such that the accuracy of the estimates of the contribution of individual proteins to the overall observed fluorescence is maximized. For the former task, the overall fluorescence intensity of a sample is measured using a plate reader and it is assumed to be a linear superposition of the intensities of the individual proteins present in it. The goal of the latter is to make the decision of which different fluorescent proteins should be used in an experiment amongst the available proteins based on the D-optimality design criterion. The developed techniques have been validated using experimental data from mixtures of different *E. coli* strains where each type of *E. coli* expressed a different fluorescent protein.

2. Methods

For the purpose of this study, *E. coli* strains, suspended in a clear media and expressing different fluorescent proteins, were mixed together to create mixtures containing up to three different fluorescent proteins, similar to what is commonly done in the field of biofilm analysis (Ma and Bryers, 2010).

2.1. Bacterial strains, plasmids and cell culture

The *E. coli* strains and the fluorescent protein plasmids used in this study and their source are listed in Table 1. For cell culture, the *E. coli* strains containing the corresponding plasmid were taken from glycerol stocks and streaked on LB agar plates supplemented with an appropriate antibiotic to maintain the plasmid (150 μg/mL erythromycin for the GFP-expressing strain and 100 μg/mL ampicillin for RFP, CFP, and YFP-expressing strains). The plates were incubated overnight at 37 °C. *E. coli* from the fresh LB agar plates were cultured overnight for 14 h in 25 ml of tryptone broth media (TB; 10 g tryptone/L H_2O and 8 g NaCl/L H_2O) with the appropriate antibiotic at 37 °C with shaking. Then, a part of the cell culture (15 mL) was centrifuged at 3000 RPM for 7 min. After visually confirming the presence of a cell pellet, the supernatant media was removed and the bacteria were resuspended in 10 mL of clear chemotaxis buffer (CB; 1 × phosphate-buffered saline, 0.1 mM EDTA (pH- 8.0), 0.01 mM L-methionine, and 10 mM DL-lactate) and diluted to different concentrations based on OD_{600} measurements.

2.2. Mixtures of different E. coli strains

The *E. coli* strains expressing different fluorescent proteins were mixed in defined ratios in a 96-well plate. Several mixtures were made containing up to three different fluorescent protein-expressing *E. coli* strains mixed in a variety of ratios. The final volume of the mixture used in each well was 150 μl. Thus, as an example, for making a 1:1:1 mixture (by volume) of *E. coli* RP437(pCM18), *E. coli* TG1(pAmCyan) and *E. coli* TG1(pZsYellow) from their individual cultures, 50 μl of each of these strain cultures were mixed together. The well plate was mixed mechanically to ensure proper mixing of the strains and their emission spectra were measured. The emission spectra of individual fluorescent proteins were also recorded for 150 μl of its culture at different concentrations (or optical densities) which were used for creating the mixtures.

2.3. Fluorescence intensity measurements using a plate reader

The fluorescence intensity measurements of individual *E. coli* strains and their mixtures were taken using a plate reader. The reason for using a plate reader is that it allows the measurement of emission intensity at various wavelengths which is not easily possible using fluorescence microscopy unless a variety of filters are used.

The maximum excitation and emission wavelengths for each fluorescent protein used are given in Table 2. The excitation

Table 1
E. coli strains and fluorescent protein plasmids.

***E. coli* strain**	**Plasmid**	**Plasmid description**
E. coli RP437 (Mao et al., 2003)	pCM18 (Hansen et al., 2001)	Green Fluorescent Protein (GFP)-expression plasmid
E. coli TG1 (Stratagene, La Jolla, CA)	pDsRed-Express (Clontech, CA)	Red Fluorescent Protein (RFP)-expression plasmid
	pAmCyan (Clontech, CA)	Cyan Fluorescent Protein (CFP)-expression plasmid
	pZsYellow (Clontech, CA)	Yellow Fluorescent Protein (YFP)-expression plasmid