

Video Article

FIBS-enabled Noninvasive Metabolic Profiling

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Abstract

In the era of computational biology, new high throughput experimental systems are necessary in order to populate and refine models so that they can be validated for predictive purposes. Ideally such systems would be low volume, which precludes sampling and destructive analyses when time course data are to be obtained. What is needed is an *in situ* monitoring tool which can report the necessary information in real-time and noninvasively. An interesting option is the use of fluorescent, protein-based *in vivo* biological sensors as reporters of intracellular concentrations. One particular class of *in vivo* biosensors that has found applications in metabolite quantification is based on Förster Resonance Energy Transfer (FRET) between two fluorescent proteins connected by a ligand binding domain. FRET integrated biological sensors (FIBS) are constitutively produced within the cell line, they have fast response times and their spectral characteristics change based on the concentration of metabolite within the cell. In this paper, the method for constructing Chinese hamster ovary (CHO) cell lines that constitutively express a FIBS for glucose and glutamine and calibrating the FIBS *in vivo* in batch cell culture in order to enable future quantification of intracellular metabolite concentration is described. Data from fed-batch CHO cell cultures demonstrates that the FIBS was able in each case to detect the resulting change in the intracellular concentration. Using the fluorescent signal from the FIBS and the previously constructed calibration curve, the intracellular concentration was accurately determined as confirmed by an independent enzymatic assay.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51200/>

Introduction

Metabolite monitoring has various applications in bioprocessing, including process development, media and feed design, and metabolic engineering. Various methods are available for concentration measurements through the use of enzymatic¹, chemical², or binding assays³. An interesting option is the use of fluorescent, protein-based *in vivo* biological sensors as reporters of the intracellular concentration of key metabolites. In ultra-low volume assays, fluorescence is a convenient tool as miniaturization actually improves the signal-to-noise ratio^{4,5} and protein-based sensors can be genetically encoded meaning that no exogenous reagents are necessary for metabolite analysis. Förster Resonance Energy Transfer (FRET) biosensors consist of two fluorescent proteins connected by a ligand binding domain. FRET is a nonradiative transfer of energy from a photo-excited donor to an acceptor fluorescent molecule located in close proximity (<100). Ligand cleavage or binding causes a conformational change in the sensor, which, in turn, induces a change in the proximity of the fluorophores, leading to a change in FRET efficiency measured by the change in the emission spectrum. FRET integrated biological sensors (FIBS) are constitutively produced within the cell line and their spectral characteristics change based on the concentration of metabolite within the cell. FIBS have fast response times making them ideal for taking measurements for dynamic models⁶. Previous applications include monitoring single⁷⁻⁹ and multiple¹⁰ metabolites and providing data on spatiotemporal distribution¹¹. FIBS can be created in two configurations: Apo-Max, where ligand binding disrupts the proximity of the fluorophores lowering energy transfer and Apo-Min, where ligand binding brings the two fluorophores into closer contact (**Figure 1**).

In this work, a protocol is presented for constructing Chinese hamster ovary (CHO) cell lines that constitutively express a FIBS for a metabolite, glucose or glutamine. A methodology is established for the calibration of the sensor *in vivo* to enable future quantitative measurements of intracellular metabolite concentration, as presented in **Figure 2**. Based on this, the intracellular concentration of glucose or glutamine can be determined in fed-batch CHO cell cultures, to which the two nutrients were added in high concentrations in-process. The results demonstrate that using the fluorescent signal from the FIBS and the previously constructed calibration curve, accurately prediction of the intracellular concentration is possible, as confirmed by an independent enzymatic assay. This method offers substantial advantages over current analytical technologies because it is noninvasive, low-cost and fast, giving a real-time signal of the FIBS that can be monitored throughout the culture.

Protocol

1. Cell Line Revival and Maintenance

1. Revive CHO cells in 9 ml CD-CHO medium supplemented with 8 mM L-Glutamine and 10 ml/L 100x hypoxanthine/thymidine supplement (complete growth medium).
2. Centrifuge at 100 x g for 5 min.
3. Resuspend the cells in 10 ml of fresh complete growth medium.
4. Remove a 1 ml sample and determine the viable cell concentration by light microscopy using the trypan blue dye exclusion method in a hemocytometer.
5. Initiate a culture at a seeding density of 3×10^5 cells/ml in 125 ml shake flasks.
6. Maintain the culture in an incubator at 37 °C, a humidified atmosphere of 5% CO₂, on an orbital shaking platform rotating at 120 rpm.
7. Subculture every 3-4 days in the complete growth medium at a seeding density of 2×10^5 cells/ml.

2. Transfection of the Cell Line with the Plasmid Containing the Biosensor Gene

1. Prepare plasmid DNA using a large-scale plasmid purification kit.
2. Maintain cells at the appropriate conditions (37 °C, 5% CO₂) 12-24 hr prior to transfection to ensure the cells are actively dividing at the time of transfection.
3. Count the cells using the trypan blue dye exclusion method, then prepare a working volume of 20 ml of cell culture in a 125 ml shake flask at a concentration of 1×10^6 cells/ml.
4. Use a suitable transfection kit for the cell line in use. The plasmid DNA to transfection reagents ratio will be dependent on the cell line and vector. It is recommended that this is optimized by testing a range of ratios within the range specified by the manufacturer prior to conducting the final transfections. Also maintain at least one negative control culture containing the cells but no DNA.
5. Incubate for 4 days in static mode and then transfer to a shaking platform rotating at 125 rpm.
6. Add the appropriate antibiotic for plasmid selection to a suitable concentration as determined by a kill curve. In this study, zeocin was added to a final concentration of 400 µg/ml for selection of transfected cells.
7. Change media at an appropriate time interval for the cell line, adding antibiotic each time, until cells in control well have died.
8. Use the most confluent wells to progress to shaking cultures.
9. Establish a cell bank by freezing the cells in cryogenic vials containing 10^7 viable cells in 1 ml of freeze mix. In this work, this consists of 92.5% growth medium and 7.5% dimethyl sulfoxide.

3. Batch and Fed-batch Cell Growth Curve

1. Follow instructions for cell maintenance above to establish triplicate cell cultures of the transfected CHO cells in 250 ml shake flasks with a working volume of 50 ml.
2. Maintain the cultures in a humidified cell incubator at 37 °C, with 5% CO₂, on an orbital shaking platform rotating at 125 rpm, and remove 4.1 ml samples from each growing culture at 24 hr intervals.
3. Use 100 µl of the sample to determine the viable cell concentration and cell viability with the trypan blue dye exclusion method.
4. Repeat until the viable cell concentration is reduced to zero.
5. Repeat for the fed-batch cell cultures supplementing with the appropriate amount of glucose or glutamine on day 6 to restore their concentrations to their initial values of 36 mM and 4 mM, respectively. It is recommended that additional control cultures are also maintained, which are to be fed with the same volume (12 ml in this case) of pure water.

4. FRET Ratio Measurements

1. Take 2 ml of the daily samples removed in step 3.2 above and centrifuge at 100 x g for 5 min at 4 °C.
2. Resuspended the cell pellet in 2 ml of ice cold phosphate buffered saline (PBS). Transfer this into a 6-well plate and add a blank sample containing no cells to one well.
3. Measure the levels of blue and yellow fluorescence immediately at an excitation wavelength of 430/435 nm and emission wavelengths of 465/435 nm (blue) and 520/510 nm (yellow).
4. Calculate the FRET ratios (ratio of yellow fluorescence detected over blue fluorescence detected).

5. Metabolite Assays

1. Take 2 ml of the daily samples removed in step 3.2 above and centrifuge at 100 x g for 5 min at 4 °C. Remove the supernatant.
2. Resuspend the cell pellet in 3 ml ice cold PBS and centrifuge again at 100 x g for 5 min.
3. Remove the supernatant and resuspend the cell pellet in 3 ml ice cold PBS as above. Sonicate the sample 5x for 3 min each at a pulse of 15 sec on and 15 sec off. At this point, the cell extracts may be frozen if desired.
4. Use an appropriate glucose assay kit as per the manufacturer's instructions on samples of cell extracts to determine the intracellular glucose concentration (see table of materials).

Note: Use appropriate standards to construct a standard curve. In this work, the concentration of standards ranged between 0-100 mM.

5. Using the readings for the standards, construct the standard curve and find the linear equation of best fit (in this case: $y = 1310.51x - 723.43$ where y is the absorbance reading and x is the glucose concentration).

- Calculate the glucose concentrations using the standard curve and taking the dilution into account. The intracellular glucose concentration can then be calculated using this equation:

$$\text{Intracellular concentration} = \frac{\text{Concentration in sample}}{\text{Viable cell concentration (cells.L}^{-1}) \times \text{Single cell volume (L)}} \quad [1]$$

For CHO cells, the single cell volume was calculated using a cell diameter of 12 μm ¹².

- Use an appropriate glutamine assay kit as per the manufacturer's instructions on samples of cell extracts to determine the intracellular glutamine concentration (see table of materials).

Note: Use appropriate standards to construct a standard curve. In this work, the concentration of standards ranged between 0-2 mM.

- As in 5.5 above, construct the calibration curve (in this case $y = 203.1x + 17.1$ where y is the absorbance reading and x is the glucose concentration)
- Using the standard curve, calculate the glutamine concentration of the samples, taking the dilution into account. The intracellular glutamine concentration can be calculated using **Equation 1** above.

Representative Results

An overview of the methodology is presented in **Figure 2**. In the work presented herein, CHO cells were transfected with the FIBS vector and stable cell lines were selected at an antibiotic pressure of 400 $\mu\text{g/ml}$ zeocin. Two separate stable cell lines constitutively expressing the glucose and glutamine sensors were therefore created. **Figure 1** depicts the configurations of the two biosensors used in this study. The glucose sensor is based on the Apo-Max principle and the corresponding plasmid is pRSET-FLIPglu600 $\mu\Delta$ 11Aphrodite. This is a fusion of the enhanced cyan fluorescent protein (ECFP) and the Aphrodite variant of the enhanced yellow fluorescent protein (EYFP) to the F16A mutant of the *E. coli* glucose/galactose periplasmic binding protein. 11 amino acid residues were removed from the linker region to improve FRET efficiency¹³. This vector was cloned into the pCDNA4/TO vector using the unique *EcoRI* and *PstI* restriction sites, which uses a Cytomegalovirus promoter and a SV40 enhancer.

The glutamine sensor is based on the Apo-Min principle (**Figure 1**) and the corresponding plasmid was derived from an insertion of the yellow fluorescent protein citrine into the *E. coli* glutamine periplasmic binding protein between amino acids 98 and 99 with an ECFP attached to the C-terminus. The structure has been rigidified by deletion of residues in the linking region, while the amino acid substitution D157N has been made to the QBP to increase affinity for glutamine, followed by several mutations to optimize FRET efficiency⁸. The glutamine FRET construct was supplied in the pUTKan plant expression vector and was digested with *Bam*HI and *Sal*I to release the insert. The latter was cloned into the corresponding restriction sites in the vector pET41a in frame with the N-terminal purification tags. The same insert was ligated into the pCDNA4/TO vector, after it had been digested with *Bam*HI and *Xho*I using the compatible sticky ends produced by *Sal*I and *Xho*I, to produce the mammalian expression vector.

Plasmid expression was confirmed by quantifying the mRNA levels of the biosensors using qRT-PCR. Both FIBS were found to be actively transcribed at 25-44 mRNA copies per cell assuming β -actin at 3,000 transcripts per cell at the mid-exponential level¹⁴.

Batch overgrow cultures of each cell line were maintained taking daily samples for the *in vivo* calibration of each FIBS. The viable cell growth profile of the two cell lines is shown in **Figure 3** and the corresponding FRET measurements and intracellular metabolite concentrations determined by enzymatic assays are presented in **Table 1**. A fluorescence plate reader was used to measure fluorescence of samples at an excitation wavelength of 430/35 nm and emission wavelengths of 465/435 nm for cyan and 520/510 nm for yellow. It was found that low cell numbers present in the first 4 days of culture led to unreliable FRET signal. Similarly, the high level of cell lysates present after day 8 of culture produced a high amount of light scattering. Only data for days 4-8 is therefore used to construct the calibration curves for the glucose and glutamine FIBS, the equations for which are shown in **Table 1**. The results show that the FIBS signal produces a reliable correlation with the intracellular concentrations between 1-5 mM for glucose and 0.3-2 mM for glutamine.

Following this and to validate the above findings, fed-batch overgrow cultures of the two cell lines were performed. A feed containing high substrate concentration (glucose in the case of the glucose FIBS and glutamine in the case of the glutamine FIBS) was supplemented on day 6 of the cell culture to elevate the extracellular concentrations of the substrate. In the case of the glucose-fed culture the extracellular glucose concentration was increased back up to 36 mM, while for the glutamine-fed culture, the glutamine concentration was increased to 4 mM. **Figure 4** shows representative results from this study. **Figures 4A** and **4C** depict the FRET ratios on each day, which clearly respond to feeding by reversing the trend they followed up to day 6. Specifically, in **Figure 4A**, the FRET ratio for the glucose FIBS CHO cell line decreases on day 7 in response to glucose addition, since the glucose FIBS follows the APO-On configuration. Similarly, in **Figure 4C**, the FRET ratio for the glutamine FIBS CHO cell line increases in response to glutamine addition, in line with the principle of APO-Off sensors.

These FRET measurements were finally used to calculate the corresponding intracellular glucose and glutamine concentrations based on the aforementioned calibration curves and the results are shown in **Figure 4B** for glucose and **Figure 4D** for glutamine. They are compared to the actual intracellular concentrations of these substrates as determined with the glucose and glutamine enzymatic assays and show adequate agreement.

Glucose FIBS			Glutamine FIBS	
Day	FRET ratio	Intracellular concentration (mM)	FRET ratio	Intracellular concentration (mM)
4	1.5	5.56	3.5	2.11
5	3.5	3.05	2.0	1.12
6	5.7	2.11	1.8	0.84
7	6.6	1.14	0.2	0.58
8	7.1	1.00	1.4	0.32

Table 1. Results for *in vivo* calibration of FRET ratio for glucose and glutamine FIBS. The calibration curves are described by the following equations that can be used to make predictions: $y = -1.232x + 8.034$ for glucose ($R^2=0.961$), and $y = 1.892x + 0.332$ for glutamine ($R^2=0.879$), where y is the FRET ratio and x is the concentration in mM.

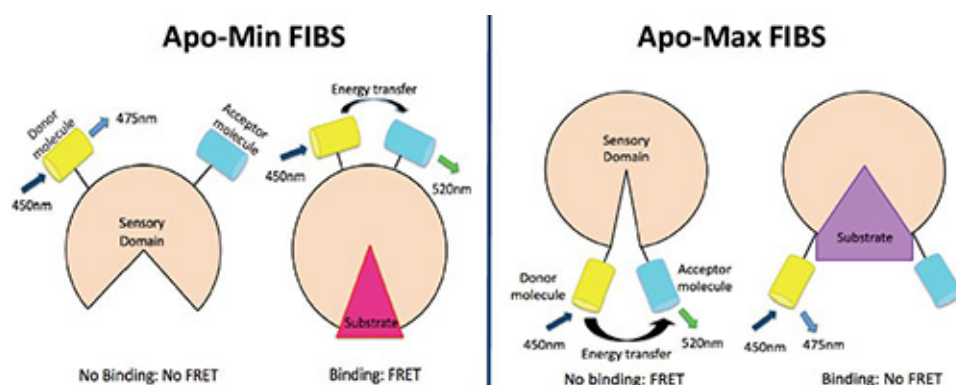


Figure 1. Principles of the FIBS used in this study. The glutamine FIBS (left) follows the Apo-Min principle, by which glutamine binds to the sensing domains and generates a conformational shift that brings both fluorescent proteins closer together, thus increasing FRET. The glucose FIBS (right) follows the Apo-Max principle, by which glucose binds to the sensing domain and generates a conformational change that separates the fluorescent proteins, thus decreasing FRET.

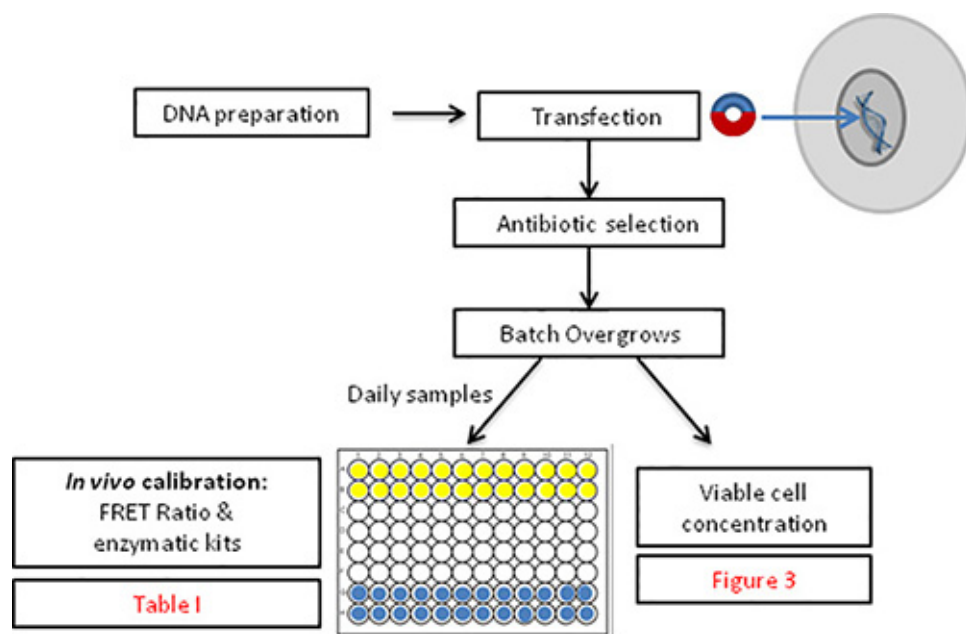


Figure 2. Overview of the methodology for FIBS calibration and subsequent noninvasive quantification of intracellular glucose and glutamine concentrations.

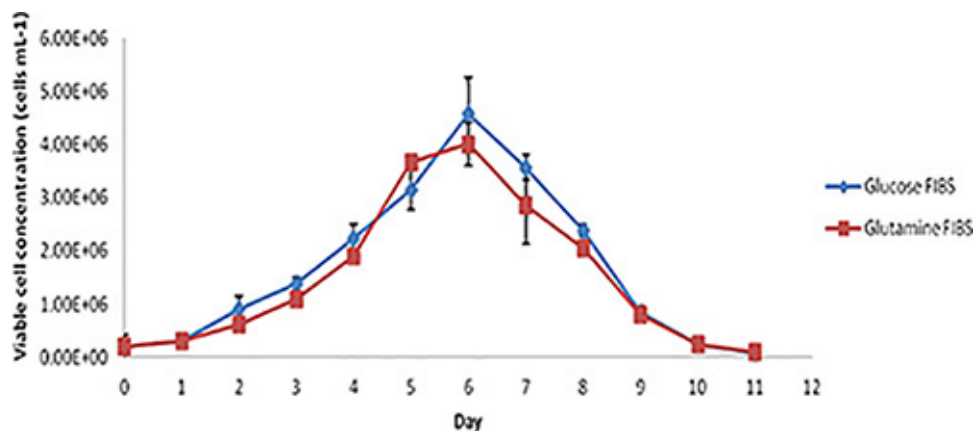


Figure 3. Viable cell concentration profile for batch overgrowth cultures of glucose FIBS- and glutamine FIBS-expressing CHO cell lines (n=2 biological repeats with three measurements per cell line).

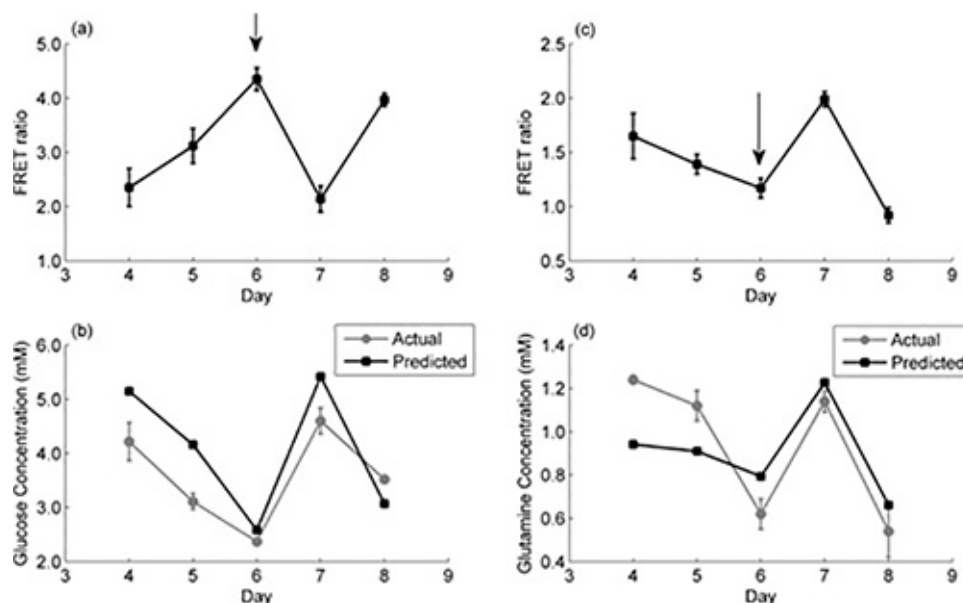


Figure 4. Fed batch culture of FIBS-producing CHO cells and corresponding FRET measurements. (a) Daily measurements of FRET ratio of fed-batch CHO cell cultures expressing glucose FIBS. The arrow indicates the addition of glucose to the cell culture. (b) Actual intracellular glucose concentration profile measured using a glucose assay kit against values predicted based on *in vivo* glucose calibration curve (Table 1). (c) Daily measurements of FRET ratio of fed-batch CHO cell cultures expressing glutamine FIBS. The arrow indicates the addition of glutamine to the cell culture. (d) Actual intracellular glutamine concentration profile measured using a glutamine assay kit against values predicted based on *in vivo* glutamine calibration curve (Table 1). In all cases, n=2 biological repeats with three measurements per cell line.

Discussion

The FIBS enable *in vivo* and *in situ* quantitation of key molecules, in this case growth-limiting nutrients, removing uncertainties arising from quenching and extraction methods. The findings suggest that there is a good correlation between the FIBS signal and the intracellular concentrations in the range of 1-5 mM for glucose and 0.3-2 mM for glutamine. In batch CHO cell culture, these concentrations are encountered in the exponential, stationary, and early decline phases. The exponential and stationary phases are the most critical in terms of the design of appropriate feeding strategies; the use of these biosensors for estimating the metabolic requirements of the cells is therefore industrially relevant. The results of the fed-batch experiment confirm the FIBS' suitability to provide estimates of intracellular glucose and glutamine concentrations. Deviations exhibited at earlier time points may have arisen because of lower viable cell numbers and/or because of interference by other metabolites, e.g. by galactose in the case of the glucose FIBS, and by other amino acids in the case of the glutamine FIBS. Specifically, it has been shown that a reduction of up to 20% in the FRET ratio can occur in the presence of aspartate, glutamate, glycine, threonine, valine, and tyrosine¹⁵. Overall, these results suggest that error in the FIBS signal is fairly low.

Although this methodology can only monitor a limited number of molecules at a time, it can provide useful information for cell line and process development, or even data acquisition for the validation of mathematical models. Given the diversity of periplasmic binding proteins, similar biosensors can be developed for an array for small molecules including other amino acids, sugars, and ions such as sulfate and phosphate¹⁶, making this a versatile method for metabolite quantification in real-time in living cells.

A key benefit of the use of FIBS is the ability to obtain online measurements potentially in a continuous fashion. The method therefore lends itself for high-throughput applications which can reduce manual handling and expedite development efforts. With the increased availability of microreactor environments with built-in functionality for fluorescence detection, it is envisaged that a FIBS-enabled metabolite profiling platform can be used for cell screening, media and process optimization, and process scale-up.

Disclosures

The authors have nothing to disclose.

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