Video Article

# FIBS-enabled non-invasive 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 non-invasively.

For metabolite monitoring, which also has applications in bioprocess development, medium formulation, and cell line engineering, various methods are available for concentration measurements through the use of enzymatic (e.g. [1]), chemical (e.g. [2]), or binding protein-based assays (e.g. [3]). 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. One particular class of *in vivo* biosensors which has found applications in metabolite quantification is based on Förster Resonance Energy Transfer (FRET) between two fluorescent proteins connected by a ligand binding domain (Figure 1). 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 models [6]. They have previously been used to monitor single (e.g. [7-9]) and multiple [10] metabolites, and provide data on spatiotemporal distribution [11].

In this paper we describe the method for constructing Chinese hamster ovary (CHO) cell lines that constitutively express a FIBS for a metabolite, in this case glucose and glutamine, and calibrating the FIBS *in vivo* in batch cell culture in order to enable future quantification of intracellular metabolite concentration. We further present data from fed-batch CHO cell cultures, where glucose- or glutamine-rich feed was supplemented, and demonstrate 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 we were able to accurately determine the intracellular concentration as confirmed by an independent enzymatic assay.

## Protocol

### 1. Cell line revival and maintenance

1. Revive CHO cells in 9 mL CD-CHO medium supplemented with 8mM L-Glutamine and 10ml/L 100X hypoxanthine/thymidine supplement (complete growth medium).
2. Centrifuge at 800 rpm for 5 minutes.
3. Discard the supernatant and 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 an improved Neubauer haemocytometer.
5. Initiate a culture at a seeding density of $3x10^5$ cells $mL^{-1}$ in Erlenmeyer 125 flasks.
6. Maintain the culture in an incubator at 37°C, a humidified atmosphere of 5% $CO_2$, 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 $2x10^5$ cells $mL^{-1}$.

### 2. Transfection of the cell line with the plasmid containing the biosensor gene

1. Prepare plasmid DNA using a large-scale plasmid purification kit (Maxi prep)
2. Maintain cells at the appropriate conditions (37°C, 5% $CO_2$) 12-24 hours 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 20mL of cell culture in an Erlenmeyer 125 flask at a concentration of $1x10^6$ cells $mL^{-1}$.

4. Use the TransIT-PRO transfection kit as per the manufacturer's instructions. 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 our study, zeocin was added to a final concentration of 400µg $mL^{-1}$ for selection of transfected cells.
7. Change media every three days (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, which 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 Erlenmeyer 250 flasks with a working volume of 50 mL.
2. Maintaining the cultures in a humidified cell incubator at 37°C, with 5% $CO_2$, on an orbital shaking platform rotating at 125 rpm, remove 4.1 mL samples from each growing culture at 24 h 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 glucose or glutamine on day 6 to bring the concentrations of these metabolites to 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 instead of nutrients.

## 4. FRET ratio measurements

1. Take 2 mL of the daily samples removed in step 3.2 above and centrifuge at 800 rpm for 5 minutes at 4°C.
2. Discard the supernatant and re-suspended 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. Take blue and yellow fluorescence measurements immediately using a fluorescence plate reader at an excitation wavelength of 430/35 nm and emission wavelengths of 465/35 nm (blue) and 520/10 nm (yellow).
4. Calculate the FRET ratios as the amount of yellow fluorescence detected divided by the amount of blue fluorescence detected.

## 5. Metabolite assays

1. Take 2 mL of the daily samples removed in step 3.2 above and centrifuge at 800 rpm for 5 minutes at 4 °C. Remove the supernatant.
2. Re-suspend the cell pellet in 3 mL ice cold PBS and centrifuge again at 800 rpm for 5 minutes.
3. Remove the supernatant and re-suspend the cell pellet in 3 mL ice cold PBS as above. Sonicate the sample 5 times for 3 minutes each at a pulse of 15 seconds on and 15 seconds off. At this point, the cell extracts may be frozen if desired.
4. Use the Amplex Red glucose/glucose oxidase assay as per the manufacturer's instructions using a 1:2 dilution of the cell extracts to determine the intracellular glucose concentration. The standards used by the authors ranged between 0 and 100 mM. Using the readings for the standards, construct the standard curve and find the linear equation of best fit using Microsoft Excel (in our case: y = 1310.51x - 723.43 where y is the absorbance reading and x is the glucose concentration). Based on readings for samples, 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:

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

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

5. Use the EnzyChrom Glutamine Assay Kit as per the manufacturer's instructions using a 1:2 dilution of the cell extracts to determine the intracellular glutamine concentration. The standards used by the authors ranged between 0 and 2 mM. As in 5.4 above, construct the calibration curve (in our case y = 203.1x + 17.1 where y is the absorbance reading and x is the glucose concentration) and use it to calculate the glutamine concentrations using the standard curve and taking the dilution into account. The intracellular glutamine concentration can be calculated using equation 1 above. Note that because the EnzyChrom kit is a coupled enzymatic assay it is necessary to add an extra set of controls in order to be able to correct for the presence of intracellular glutamate.

## 6. Representative Results

An overview of our methodology is presented in Figure 2. In the work presented herein, we transfected CHO cells with the FIBS vector and stable transfectants were selected using 400 µg/mL Zeocin, creating two separate stable cell lines constitutively expressing the glucose and glutamine sensors, respectively. Figure 1 depicts the configurations of the two biosensors used in this study. The glucose sensor is based on the Apo-On principle and the corresponding plasmid is pRSET-FLIPglu600µΔ11Aphrodite (Addgene number 13569). 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. It has been further modified by deleting 11 amino acid residues in the linker region to optimise the FRET efficiency [13]. This vector was cloned into the pCDNA4/TO vector using the unique *Eco*RI and *Pst*I restriction sites, which uses a Cytomegalovirus promoter and a SV40 enhancer.

The glutamine sensor is based on the Apo-Off 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 made to the QBP to increase affinity for glutamine, followed byseveral mutations to optimise FRET efficiency [8]. The glutamine FRET construct was supplied in the pUTKan plant expression vector and was digested with *BamH*I and *Sal*I to release the insert which was subsequently cloned into the corresponding restriction sites in the vector pET41a in frame with the N-terminal purification tags. The mammalian expression vector was created by ligating the same insert into the pCDNA4/TO vector which had been digested with *BamH*I and *Xho*I, taking advantage of the compatible sticky ends produced by *Sal*I and *Xho*I.

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 level [14].

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 2. All fluorescence measurements were made in a Tecan Infinite 200Pro fluorescence plate reader using an excitation wavelength of 430 / 35 nm and emission wavelengths of 465 / 35 nm (cyan) and 520 / 10 nm (yellow). FRET measurements before day 4 of the cell culture are not reliable because of low cell numbers. Similarly after day 8 the high degree of cell lysis results in a high amount of light scattering. We therefore only used data for days 4 to 8 to construct our calibration curves for the glucose and glutamine FIBS, the equations for which are shown in Table 2. The results show that the FIBS signal is best correlated with the intracellular concentrations between 1 and 5 mM for glucose and 0.3 and 2 mM for glutamine.

Following this and to validate our findings, we performed fed-batch overgrow cultures of the two cell lines, 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 brought 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.

We finally used these FRET measurements to calculate the corresponding intracellular glucose and glutamine concentrations based on the aforementioned calibration curves and the results are shown in Figures 4B for glucose and 4D for glutamine. They are compared to the actual intracellular concentrations of these substrates as determined with the Amplex Red and EnzyChrom 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 2.** 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.

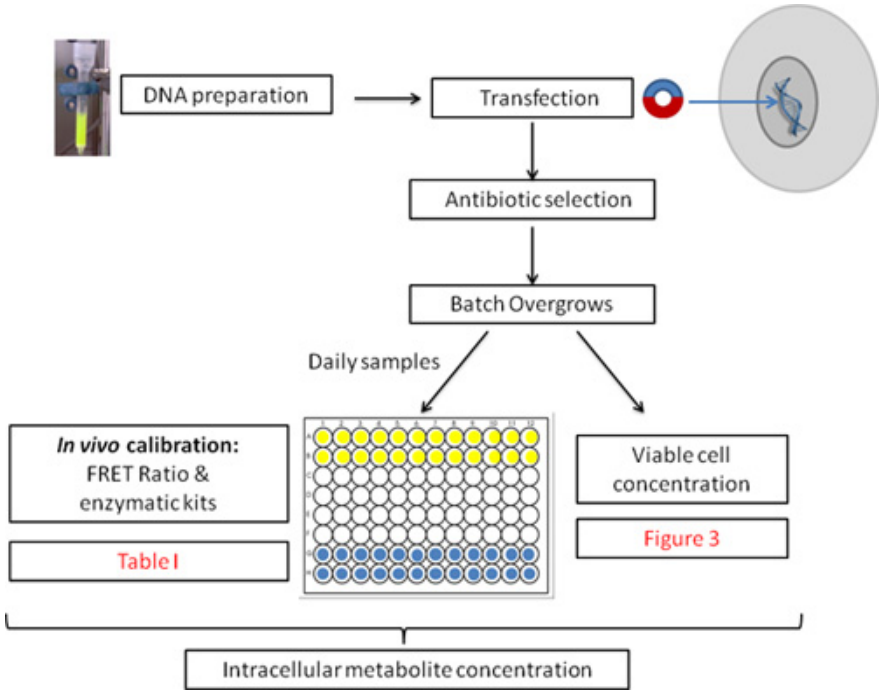


**Figure 1.** Principles of the FIBS used in this study. The glucose FIBS (left) follows the Apo-On principle, by which glucose binds to the sensing domain and generates a conformational change that separates the fluorescent proteins, thus decreasing FRET. The glutamine FIBS (right) follows the Apo-Off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

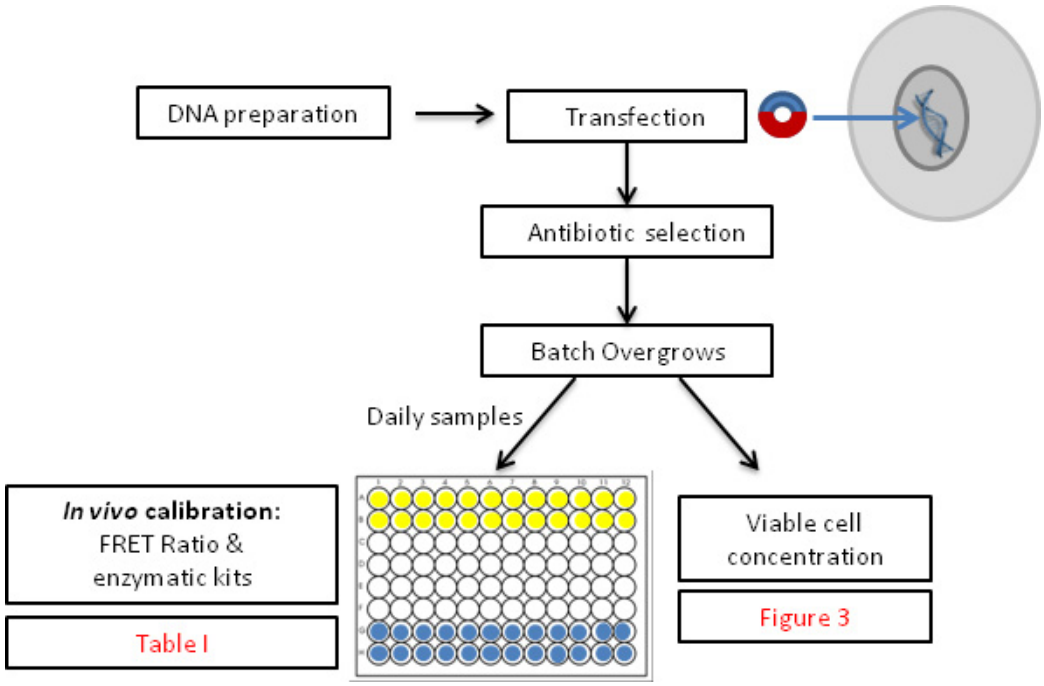


**Figure 2.** Overview of the methodology for FIBS calibration and subsequent non-invasive quantification of intracellular glucose and glutamine concentrations.

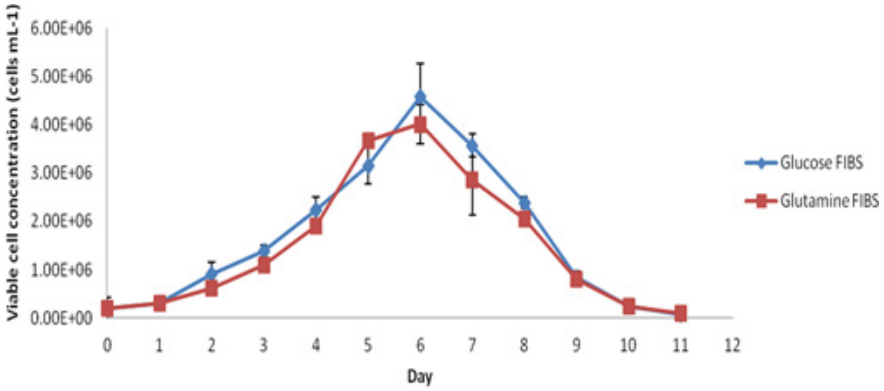


**Figure 3.** Viable cell concentration profile for batch overgrow cultures of glucose FIBS- and glutamine FIBS-expressing CHO cell lines (n=2 biological repeats with three measurements per cell line).

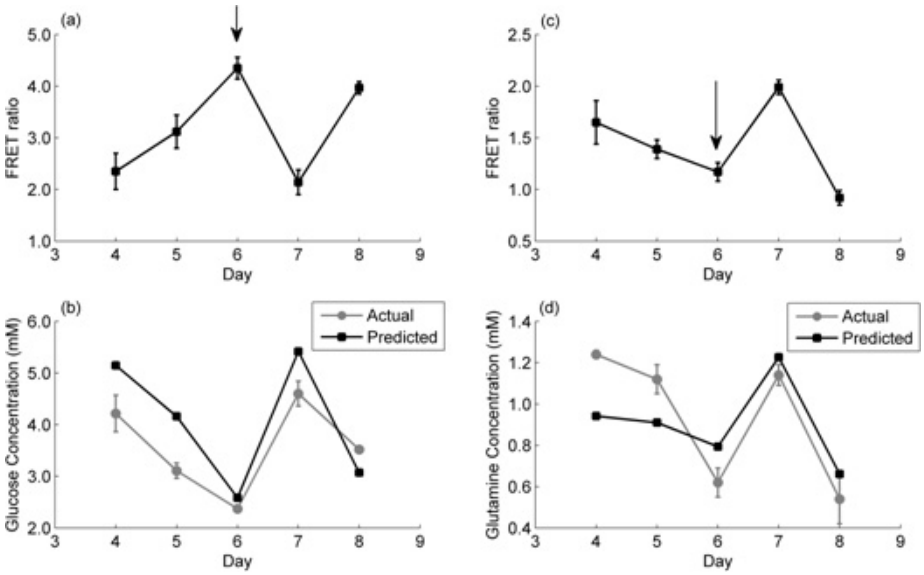


**Figure 4.** Fed batch culture of FIBS-producing CHO cells and corresponding FRET measurements. (a) Daily measurements of FRET ratio of fed-batch CHO cell cultures expressing glucose FIBS. The arrow indicates the addition of glucose to the cell culture. (b) Actual intracellular glucose concentration profile measured using the Amplex Red glucose assay kit against values predicted based on *in vivo* glucose calibration curve (Table 2). (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 the EnzyChrom glutamine assay kit against values predicted based on *in vivo* glutamine calibration curve (Table 2). 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 our case growth-limiting nutrients, removing uncertainties arising from quenching and extraction methods. Our findings suggest that the FIBS signal is best correlated with the intracellular concentrations between 1 and 5 mM for glucose and 0.3 and 2 mM for glutamine. These ranges correspond to the exponential, stationary, and early decline phases of the CHO cell culture, which are the most important in terms of elucidating metabolic requirements in a bioprocessing context. 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, we have shown that a reduction of up to 20% in the FRET ratio can occur in the presence of aspartate, glutamate, glycine, threonine, valine, and tyrosine [15]. 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 sulphate and phosphate [16], 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, such as the Biolector system (m2p-labs, Germany), we envisage our FIBS-enabled metabolite profiling platform being used for cell screening, media and process optimization, and process scale-up.

## Disclosures

No conflicts of interest declared.

## Acknowledgements

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