

Opportunities for bioprocess monitoring using FRET biosensors

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Abstract

Bioprocess monitoring is used to track the progress of a cell culture and ensure that the product quality is maintained. Current schemes for monitoring metabolism rely on offline measurements of samples of the extracellular medium. However, in the era of synthetic biology, it is now possible to design and implement biosensors that consist of biological macromolecules and are able to report on the intracellular environment of cells. The use of fluorescent reporter signals allows non-invasive, non-destructive and online monitoring of the culture, which reduces the delay between measurement and any necessary intervention. The present mini-review focuses on protein-based biosensors that utilize FRET as the signal transduction mechanism. The mechanism of FRET, which utilizes the ratio of emission intensity at two wavelengths, has an inherent advantage of being ratiometric, meaning that small differences in the experimental set-up or biosensor expression level can be normalized away. This allows for more reliable quantitative estimation of the concentration of the target molecule. Existing FRET biosensors that are of potential interest to bioprocess monitoring include those developed for primary metabolites, redox potential, pH and product formation. For target molecules where a biosensor has not yet been developed, some candidate binding domains can be identified from the existing biological databases. However, the remaining challenge is to make the process of developing a FRET biosensor faster and more efficient.

Current technology for bioprocess monitoring

The use of biological entities for the production of useful compounds dates back thousands of years when yeast was first harnessed for fermentation. In our daily lives, we encounter many such products from the enzymes in our washing powder to the vitamin supplements we take in the morning. Biological production systems have also been developed to produce the medications used to fight human diseases ranging from small-molecule therapies to protein therapeutics and whole-cell therapies.

Biological systems are inherently complex, but economical manufacturing processes require reproducibility. The solution to this paradox has been to identify key indicators of the cell-culture performance and to monitor these so that they stay within the limits known to result in the maximum yield of a high-quality product. In this way, even though many of the underlying cellular processes remain a 'black box', the quality of the final product is ensured². Monitoring is also used to ensure that the growth conditions remain constant

and that the cells have adequate access to nutrients. The accumulation of the product may also be measured in order to determine when it should be harvested [1].

The identification of which indicators correlate most strongly with productivity is an active area of research and varies on a case-by-case basis. The list can include the main nutrients for the cell type being used: carbon, nitrogen and trace element sources, metabolic waste products that can affect cell function, and signalling molecules that can suggest cellular stress. Nutrient utilization can be used to predict the rate of cell growth, the rate of product formation and to determine when cells should be fed in fed-batch processes. Waste product accumulation can provide indications of metabolic limitations. For example, lactate accumulation can suggest a need for improving oxygen transport to the culture. The concentration of the product, or its precursors, can be measured directly in order to have a more direct assessment of productivity [2].

Physical parameters such as temperature, pH and dissolved oxygen tension are monitored and controlled online throughout the bioprocess. A probe is inserted into the bioreactor that measures the parameter in question and reports the measurement to a computer system that analyses the data and then decides if any control action is needed to bring the value back in line with its desired value. For example, a temperature probe monitors the current temperature in the bioreactor and sends a signal to a computer that determines whether the flow rate of cooling water needs to be increased

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Abbreviation used: CHO, Chinese-hamster ovary.

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²Current initiatives by regulatory bodies such as the European Medicines Agency and the U.S. FDA (Food and Drug Administration) are trying to change this by introducing more rational frameworks to understand the link between process conditions and product quality. The application of one such initiative, Quality-by-Design, to the production of recombinant therapeutics has been reviewed in [50].

(because the temperature is above the set point) or decreased (because the temperature is below the set point). Similar systems can be used to control the addition of acid or base for adjusting pH and the flow-rate of air into the bioreactor for increasing the dissolved oxygen tension [3].

For other compounds, the majority of measurements employed on the industrial scale are aimed at the extracellular environment and are achieved by removing a sample of the growth medium from the bioreactor and analysing it offline [1]. For small molecules (nutrients and the resultant metabolites), HPLC is the most common technique. A number of 'analysers' have been developed, which measure the concentration of a handful of metabolites known to be important from a single sample. This list includes glucose, glutamine, ammonia, lactate and carbon dioxide ($p\text{CO}_2$) that are associated with the health and productivity of multiple types of cells. Depending on the system and the product, additional case-specific metabolites may also be monitored using HPLC or another appropriate analytical technique (GC, MS etc.) [2,3].

The difficulty with such analyses is that a time delay ensues between when the sample was taken and when the information can be acted upon. This means that if intervention is needed to correct the culture conditions (e.g. if cells have run out of the primary carbon source), the situation will continue to deteriorate before the results of the analysis are available. In addition, manual handling steps are subject to a higher degree of error than that of an automated system. Thus there is a drive towards developing automated online measurement systems [1]. A number of such systems have been described recently, although the majority have not yet been adopted in industry. The challenge is to develop low-cost reliable solutions for online monitoring of all indicators of interest. Ideally, these solutions should be easily adapted to measuring new targets in different cellular systems. One potential solution is to develop biological sensors for parameters of interest. These have the added advantage that they can be expressed within the cell culture, providing a non-destructive intracellular measurement which, in some cases, is more directly indicative of the biological process.

Biosensors

In general, a biosensor is any device which uses a biological recognition element to perform a measurement on a sample. The device must also then convert that measurement into a reportable signal (electrical, chemical, colorimetric, fluorescent, luminescent etc.). A variety of different classes of biosensors have been developed utilizing different biological macromolecules as recognition elements. The components can be used as purified parts of a mostly man-made device, for example, with blood glucose monitors that use enzymes as their recognition element. Alternatively, the biosensor can be encased entirely within a biological entity, a genetic circuit that measures the intracellular concentration of a metabolite within its host or a whole-cell biosensor that measures its

extracellular environment and reports the concentration of a molecule there [4].

Among the advantages of biosensors is the specificity of detection. Many biological recognition elements, particularly protein active sites or ligand-binding domains, have evolved to be highly specific for their target molecules. In addition, depending on the system, biosensors can be very cost competitive. A single whole-cell biosensor can be grown in relatively cheap growth medium to produce millions more biosensors, each of which can be propagated further. Biosensors that are genetically encoded offer a further advantage in that they can report on the intracellular environment of the cell of interest without the need for sampling and sample destruction. This makes them amenable to use in low-volume assays where repeated measurements of the same cell can be performed over time.

Biosensors can be distinguished based on the type of recognition element or the transduction process to produce the signal. DNA, RNA and proteins can all be used as recognition elements. The processes of transcription, translation, nucleic acid processing, protein conformation and enzymatic activity have all been exploited as signal transduction processes. One interesting class of biosensors for quantification of target molecule concentrations are protein-based biosensors that utilize FRET as the signal transduction process. Readers interested in learning more about other types of biosensors can refer to [4,5] for reviews.

FRET is a non-radiative energy transfer between two fluorophores that are in close proximity [6]. For FRET to occur, the emission spectrum of the donor fluorophore must overlap with the excitation spectrum of the acceptor fluorophore. The higher the degree of overlap, the higher the efficiency of the energy transfer and the stronger the biosensor signal. FRET efficiency is also distance-dependent: the closer the fluorophores are the more likely energy is to be transferred into excitation of the acceptor, rather than be emitted by the donor.

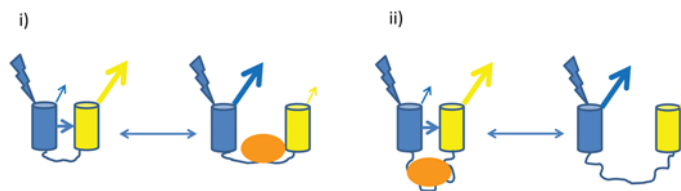
To utilize FRET as a biosensor output signal, it is necessary to cause the proximity between the fluorophores to change as a function of the presence or absence of the target molecule to be detected. This strategy relies on inducing a conformational change in the biosensor upon molecular recognition (Figure 1). Based on the ratio of emissions corresponding to the donor and acceptor, it is then possible to estimate the concentration of the molecule of interest. One significant advantage of FRET over other types of signal transduction is that the output is inherently amenable to normalization because it relies on the relative strength of two emissions. Thus small differences in experimental set-up or in biosensor concentrations should be normalized away during the calculation of the FRET ratio.

Examples of existing FRET sensors and their applications

FRET biosensors can be constructed from any type of biological macromolecule, but, to date, the majority of

Figure 1 | A FRET biosensor requires a conformational change that alters the distance between the fluorophores in the presence of the target molecule

The conformational change can be accomplished in two ways: (i) the binding of the target molecule by the binding domain moves the fluorophores apart, decreasing the FRET ratio as the concentration of the target increases, or (ii) the binding pocket collapses around the target molecule, bringing the fluorophores closer together and increasing the FRET ratio as the concentration of the molecule increases.



sensors, which are useful in a bioprocessing context have been constructed from proteins. The general structure consists of the genes for two fluorescent proteins with overlapping spectra fused to the gene for a protein that binds the molecule of interest. When expressed within cells, the biosensor protein is produced constitutively and fluctuations in the target molecule concentration lead to changes in the spectral signal.

Because of the requirement for a large conformational change upon binding, the selection of the molecule-binding domain is key to developing a biosensor that functions well. Many biosensors have been developed utilizing proteins that are known to produce a conformational change upon binding their target molecule, such as bacterial periplasmic binding proteins (e.g. [7–10]) or small-molecule-responsive transcription factors [11,12].

It can be difficult to predict the three-dimensional conformation that the fusion protein will adopt and it is currently very hard to predict the conformational changes as a result of ligand binding. Thus most examples to date rely on protein engineering techniques to improve the signal-to-noise ratio using linker engineering [13], truncation [9], circular permutation [14] and/or domain insertion [9] followed by screening for biosensors with improved characteristics. The current state-of-the-art in FRET biosensor engineering has been reviewed recently in [6].

Metabolites

Figure 2 shows a schematic representation of central metabolism and amino acid biosynthesis, features of metabolism that are relatively conserved across organisms. It may be of interest to monitor molecules in these biochemical pathways in order to predict biomass accumulation and/or to understand when cells might be running out of an essential nutrient. Several FRET biosensors have been developed to monitor metabolites within these pathways (Figure 2, red boxes). The targets that can currently be detected include glucose [7,9,15–20], glutamine [7,14,21,22], glutamate [9,14,23–25], tryptophan [11,26], arginine [14,27], histidine [14], leucine

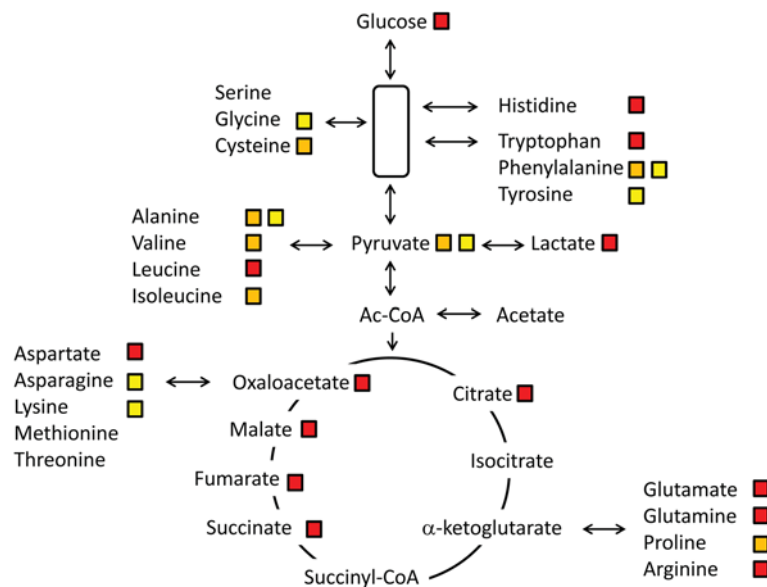
[14], lactate [12], citrate [28] and other C_4 carboxylates [8], as well as the associated cofactor ATP [20,29]. For other metabolites, candidate binding domains can be identified by searching one of several databases or the relevant literature. For example, the PDB can be used to identify crystal structures of useful bacterial periplasmic binding proteins (Figure 2, orange boxes) or databases such as EcoCyc can be used to find small-molecule-responsive transcription factors (Figure 2, yellow boxes) that could serve as starting points for FRET sensor development. Additional biosensors have been developed for other sugars, including maltose [10,13], sucrose [30], ribose [8,31] and disaccharides [8], as well as elements such as divalent metal ions [32–34] and inorganic phosphate [35] that may be of interest in certain systems. A comprehensive database of protein-based biosensors is maintained by the Carnegie Institution for Science [6].

The use of FRET biosensors to directly quantify the intracellular concentration of metabolites has been demonstrated in several types of cells [7,15–20]. Glucose is the most common metabolite to be examined quantitatively, in part because FRET biosensors for glucose have been developed with a very high signal-to-noise ratio. Detailed protocols have been published that describe the steps necessary to gain reliable quantitative information [15,17,36].

One study examined the intracellular concentrations of glucose and glutamine in a bioprocessing context using fed-batch culture of CHO (Chinese-hamster ovary) cells. CHO cells are the workhorses for recombinant protein therapeutic production in industry, and fed-batch strategies are used to prolong the life and productivity of the culture. In this example, the biosensor signal was calibrated against a standard enzymatic assay for each metabolite in order to correlate the signal to the actual metabolite concentration [7]. The resulting calibration curve was then used to predict the metabolite concentrations after feeding. Subsequent work showed that this calibration curve could be used to predict metabolite concentrations in fed-batch culture to within approximately 20% (A. Behjousiar, K.M. Polizzi and C. Kontoravdi, unpublished work).

Figure 2 | A simplified schematic of the generic metabolism of glucose and amino acids

Red boxes indicate metabolites for which a FRET sensor has been constructed. Orange boxes indicate metabolites where a crystal structure for a periplasmic binding protein for this molecule has been deposited in the PDB. Yellow boxes indicate a small-molecule-responsive transcription factor exists in the EcoCyc database.



Redox environment

FRET biosensors have also been used to monitor the redox environment within cells by placing cysteine residues in the linker region between the fluorophores (e.g. [37,38]). Although these biosensors have yet to be used in a bioprocessing context, redox metabolism, particularly the availability of GSH, which is a marker for oxidative stress, has an impact on cellular productivity [39]. Other ratiometric (non-FRET) biosensors have been developed by the strategic placement of disulfide bonds within GFP [40]. These have been used to monitor the redox potential in the ER (endoplasmic reticulum), which has been linked to protein secretion stress [41]. Biosensors capable of monitoring H_2O_2 , which can be a by-product of certain enzymatic reactions, have also been developed [42].

Other analytes

Biosensors of intracellular pH have been developed both as ratiometric FRET sensors [43,44] and in single fluorescent protein (non-ratiometric) versions [45]. In both cases, the spectral signal change exploits the fact that certain variants of fluorescent proteins (e.g. EYFP) are naturally pH-sensitive [46]. Intracellular pH can have a strong influence on protein trafficking and glycosylation [47] and so it may be desirable to monitor this independently from extracellular pH during bioprocessing.

Another biosensor design that utilizes the inherent chemistry of the fluorophore is FluBO, a ratiometric biosensor based on FRET between an oxygen-sensitive

fluorescent protein (most GFP derivatives require oxygen for chromophore maturation) and a hypoxia-tolerant fluorescent flavoprotein [48]. In this case, since the fluorescence of the canonical fluorescent protein is dependent on the oxygen level, so is the FRET efficiency.

Finally, in the area of therapeutic protein production, FRET biosensors have been developed that are capable of quantifying the concentration of a user-defined monoclonal antibody. This sensor is constructed from two fluorophores that have been engineered to have a high affinity for heterodimer formation, connected by a linker that contains two antigen epitopes recognized by the antibody of interest. The sensor exists in two conformational states. In the absence of the target antibody, the fluorophores dimerize and a high FRET signal occurs. In the presence of the target antibody, the antibody binds the antigen epitopes, forcing the fluorophores apart and reducing the energy transfer. This biosensor could be useful as a substitute for ELISA for rapid determination of antibody concentration. Interestingly, it could also be used as a high-throughput screening tool to identify new antibodies that bind a target antigen [49].

Conclusions and future perspectives

There has been an increase in the number of biosensors developed in recent years, fuelled in part by the increase in the use of biotechnology in product manufacturing. With the advent of synthetic biology and its engineering methodologies, this is expected to increase even further. If synthetic biologists are designing novel biological entities as production agents, it stands to reason that genetic circuitry

for biosensing could be incorporated as part of the system design. This would allow monitoring during production as described for traditional bioprocesses. Taken a step further, the sensors could be linked to 'actuators' which could take action based on the concentration of the target of interest (i.e. increase or decrease the expression of transporters or biosynthetic genes in response to the signal generated). Such self-correcting systems would be useful for developing robust bioprocesses that require minimal human intervention.

One of the biggest remaining challenges in the application of biosensors to bioprocess monitoring is the rapid development of biosensors for new targets. Currently, there are no universal rules for the design of a well-functioning fusion protein, and the development of each sensor is a bespoke process that usually involves one or more protein engineering steps. This severely limits the pace at which new sensors can be developed. Whereas comparing the resulting biosensors from several studies does seem to provide some guidance (e.g. truncating linker regions to reduce flexibility seems to increase the maximum FRET change achieved), far more knowledge is needed in order to streamline the process. In the future, it would also be useful to develop new computational tools which are able to consider both ligand binding and the resulting conformational changes in order to assist with biosensor design.

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