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Broadening Horizons and Teaching Basic Biology Through Cell-Free Synthesis of Green Fluorescent Protein in a High School Laboratory Course

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Abstract Cell-free protein synthesis (CFPS) has emerged as a practical method for producing a broad variety of proteins. In addition, the direct accessibility to the reaction environment makes CFPS particularly suitable as a learning vehicle for fundamental biological concepts. Here, we describe its implementation as a teaching tool for a high school laboratory course. Ninety students in a biotechnology class used CFPS to study the effects of the concentrations of amino acids, cell extract, DNA, and the energy source on accumulation of active super-folder green fluorescent protein. Students estimated product concentrations simply by comparing solution colors to a printed green color gradient. This simple and inexpensive method allows for immediate measurements, and 26 of the 30 groups observed measurable product concentrations within 60 min. These student-generated data were then discussed to illustrate concepts of data analysis such as outliers and standard deviation. We also combined the laboratory experience with a visit to a university campus that included a laboratory tour and a college-style lecture. Our overall objective was to excite the students about the scientific enterprise and to instill a sense of personal relevance and attainability so that these students could realistically consider technical careers.

Keywords High school · Cell-free protein synthesis (CFPS) · PANOx SP · *Escherichia coli* · sfGFP · Green fluorescent protein · Transcription–translation

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Introduction

Proteins perform crucial tasks within biological systems, thanks to their vast diversity in structure and function. They provide structural support for all living cells, catalyze most of the known biological reactions, and transport other molecules within organisms. As a result, their proper synthesis is essential. Since the advent of recombinant DNA technology, they also comprise an important class of pharmaceuticals; today protein therapeutics represents a multi-billion dollar industry. Recombinant protein synthesis is typically conducted using living cells that harbor the gene encoding the protein, thereby making its real-time monitoring and quantification extremely difficult.

Cell-free protein synthesis (CFPS) has been under development in the Swartz laboratory for over a decade. Since the inception of the research program at Stanford, the advances made in the laboratory have enabled the efficient

cell-free production of many different types of proteins. Amino acid stabilization (Calhoun and Swartz 2006) and a natural chemical environment as well as the activation of central metabolism (Jewett et al. 2008; Jewett and Swartz 2004) were critical in producing high concentrations of these proteins from inexpensive energy sources. The key to most of these advances was the open nature of the CFPS platform that allows direct access to the protein synthesis and folding environment. We reasoned that this same feature would allow CFPS to be an effective teaching tool for illustrating basic concepts in biochemistry and metabolism in a way that is simply not possible with living systems.

We therefore designed a relatively simple experimental protocol so that beginning students could rapidly and easily observe the effects of fundamental influences on the complex process of protein synthesis. The protocol investigated the effects of varying the concentrations of the key building blocks (amino acids), the information source (DNA), the energy source (in this case, phosphoenolpyruvate, PEP), as well as the catalysts provided by the cell extract. To provide immediate feedback, we opted for the rapid production of a green fluorescent protein so that synthesis rates and total accumulation could be determined simply and inexpensively by visual inspection within a standard 90-min laboratory period. To facilitate implementation, we also showed that quantitative data could be obtained simply by comparing the color of the reaction solution to a printed color gradient.

In this article, we describe the development and initial implementation of this laboratory protocol in the biotechnology class at Sequoia High School in Redwood City, CA. The class consisted of 90 students, almost all of whom were 11th- and 12th-year students. Without previous hands-on training, these students prepared the stock solutions to adjust their experimental variable, mixed reagents to initiate the cell-free reaction, and then observed product formation. In the following class period, the students analyzed the data in depth to see which variables had the largest effect on protein production. To supplement and motivate the hands-on laboratory experience, we also conducted a university field trip that included a college-style lecture and a laboratory tour. We believe that, in addition to providing a realistic research experience and reinforcing concepts related to protein synthesis, this laboratory exercise helped to increase the students' confidence in their technical abilities, excite them about the scientific enterprise, and encourage them to continue studying molecular biology or biotechnology in college and to pursue a science career.

Learning Objectives

We sought to design the experiment so that the students will have a better understanding of:

- The importance of the following factors for protein synthesis and cell growth: (a) information exchange, (b) substrate supply and utilization, (c) energy supply, and (d) the function of complex catalysts such as the ribosome in biological systems.
- Protein production mechanisms, i.e., transcription, translation, and protein folding.
- CFPS reactions and the components involved therein.
- The research process, especially, variable testing, analyzing results, and formulating future plans by combining their own results with those from other groups.
- Data analysis, in particular, how one distinguishes representative ("good") data from outliers.

Materials and Methods

Reagent Preparation

All of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

The PANOx SP (PEP, Amino Acids, NAD, Oxalic Acid, Spermidine and Putrescine) cell-free system (Jewett and Swartz 2004) was used to produce super-folder green fluorescent protein (sfGFP) (Cabantous et al. 2005; Pédelacq et al. 2006), which is a fast-folding and robust variant of the green fluorescent protein. 20 µl of the CFPS reaction solutions included, unless otherwise specified, 10 mM ammonium glutamate, 20 mM magnesium glutamate, 175 mM potassium glutamate, 1.2 mM ATP, 0.86 mM each of CMP, GMP, and UMP, 10 mM dibasic potassium phosphate, 34 µg/ml folinic acid, 170.9 µg/ml *E. coli* tRNAs (Roche Applied Science, Penzberg, Germany), 33 mM phosphoenolpyruvate (PEP) (Roche Applied Science), 1.5 mM spermidine, 1 mM putrescine, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A, 2.7 mM sodium oxalate, 2 mM each of the 20 amino acids, 100 µg/ml T7 RNA polymerase, 6 nM pY71sfGFP plasmid, and 0.25 volume of *E. coli* S30 extract. The glutamate salts were diluted from a 10-fold concentrated (10×) solution. Similarly, a 10× NT/MP Master Mix solution contained the ATP, the three NMPs, folinic acid, and the *E. coli* tRNAs and was adjusted to pH 7.3 with 100 mM dibasic potassium phosphate. The amino acids were diluted from a stock solution, which contained a 50 mM concentration of each amino acid. This stock solution was prepared by adding the amino acids in the following order (given in their one-letter code): R, V, W, F, I, L, C, M, A, N, D, E, G, Q, H, K, P, S, T, Y. During the preparation, it was ensured that each amino acid was dissolved before the addition of the next, except tyrosine, which is added last and remains suspended in the solution. The pH of the solution was adjusted to 7.1 with potassium

hydroxide (KOH). The pH of the PEP and spermidine stock solutions was also adjusted to 7.2–7.3 with KOH.

The S30 cell extract was prepared from the KC6 strain (K12A19Δ*speA*Δ*tnaA*Δ*sdaA* Δ*sdaB*Δ*gshA*Δ*tonA*Δ*endA-met*$^{+}$) described in [12]. KC6 cells were grown on defined medium in a 10-l Biostat C fermentor (Sartorius Stedim, Melsungen, Germany) as described by Zawada and Swartz (2005). The specific growth rate was 0.96 h^{-1}. After reaching an OD_{600} of 35, the culture was cycled through a cooling coil until the temperature dropped to 12–15 °C and then was harvested and centrifuged at 5,000*g* and 4 °C for 30 min. The cells were resuspended in cold S30 buffer (10 mM Tris acetate, pH 8.2, 60 mM potassium acetate, 14 mM magnesium acetate) and centrifuged for a total of three washes, after which they were stored at −80 °C. The frozen cell paste was resuspended in 1 ml of S30 buffer per gram of wet cell weight and passed once through an Emulsiflex C-50 homogenizer (Avestin, Ottawa, Ontario, Canada) at 17,500–22,000 psi. The resulting lysate was centrifuged twice at 30,000*g* and 4 °C for 30 min each. The clarified lysate was diluted by adding 0.2 ml of S30 buffer per ml of lysate, wrapped in aluminum foil, and incubated on a rotary shaker at 120 rpm and 37 °C for 80 min. The lysate was then pipetted into 6,000–8,000 MWCO Spectra/Por dialysis bags (Spectrum Laboratories, Rancho Dominguez, CA) and dialyzed against cold S30 buffer (100 × volume differential per dialysis) at 4 °C for 30 min each for a total of four buffer exchanges. Finally, the cell extract was centrifuged at 10,000*g* and 4 °C for 20 min and the supernatant was aliquoted, flash-frozen, and stored at −80 °C.

Plasmid pY71sfGFP encodes the super-folder green fluorescent protein (sfGFP) sequence between the T7 promoter and T7 terminator. This plasmid was generated from the pY71sfGFP-T216unAA plasmid (Bundy and Swartz 2010) by removing the glycine codon and the amber stop codon in the loop before the terminal beta sheet using the QuikChange® site-directed mutagenesis protocol (Agilent Technologies, Santa Clara, CA). The pY71sfGFP plasmid was subsequently purified using the Qiagen Plasmid Maxi Kit (Valencia, CA). T7 RNA polymerase was prepared as described in Li et al. (1999) with modifications in Yang et al. (2012).

On the day of the CFPS experiment, different twofold concentrated (2×) premixes were prepared for each group, in which the components to be investigated by each group were omitted. For example, the 2× premix for the AA groups included all of the ingredients at twice the final concentration except the amino acids. Since PEP complexes with magnesium (Kim et al. 2006), the 200 mM PEP stock solution also contained 125 mM magnesium glutamate to minimize changes to the [PEP] to [Mg^{2+}] ratio. Therefore, the 2× premix for the PEP groups was formulated without PEP or magnesium glutamate. The premixes, along with the cell extract and the "blank" cell-free reaction solutions, were transported from the laboratory at Stanford to the biotechnology classroom on ice.

Generation of the sfGFP Color Gradient

The color gradient was generated prior to the laboratory course. 20 µl CFPS reactions containing 5 µM L-[^{14}C(U)]-leucine (PerkinElmer, Waltham, MA) and the mentioned reagents were incubated at 37 °C for 0.5, 1, 1.5 and 3 h. Immediately after the protein yields were measured by liquid scintillation counting (Calhoun and Swartz 2005) (described in detail in the next paragraph), these reaction solutions were diluted with "blank" cell-free reaction solutions (which contain all the reagents except DNA and thus produce no sfGFP) to create mixtures ranging from 200 to 1,000 µg/ml sfGFP in 100 µg/ml increments. Panels colored in different shades of green were created to match the color of each of these solutions. (Generation of the panels is described in detail in the Supplementary Material.) The panels in the sfGFP color gradient were then labeled with the soluble sfGFP concentrations as determined by measuring ^{14}C-leucine incorporation.

Quantification of Radiolabeled sfGFP

The CFPS reactions were first stopped by cooling the sample tubes in a −20 °C freezer for 4 min. From each reaction solution, a 4-µl sample was spotted onto two of three Whatman MM filter papers (Whatman, Springfield Mill, United Kingdom); these papers were used to measure the total protein produced. The remaining CFPS solution was centrifuged at 20,800 g and 4 °C for 15 min. 4 µl of the supernatant was spotted on the third Whatman MM filter paper and the papers were dried either overnight on the laboratory bench or for 1 h under an incandescent light bulb. After drying, the paper with the soluble protein and one of the two papers containing the total protein were submerged in ice-cold 5 % w/v trichloroacetic acid (TCA) for 15 min to precipitate the proteins on the filter paper. This incubation step was repeated twice with fresh 5 % TCA for a total of three times. The TCA-washed papers were then rinsed with deionized water and dried under a 100 watt light bulb for 1 h. Radioactivity from all of the filter papers was quantified using a Beckman LS 3801 liquid scintillation counter (Beckman Coulter, Brea, CA). Background radioactivity was determined by applying the same protocol to "blank" reactions, and this quantity was subtracted from the protein radioactivity measurements. Total and soluble sfGFP concentrations were calculated from the radioactivity measurements using the following general formula:

$$[\text{protein}] = \frac{\text{washed counts}}{\text{unwashed counts}} \times \frac{[\text{leucine}] \times \text{MW}_{\text{protein}}}{\#\,\text{leucines per protein} \times 1,000} \quad (1)$$

where washed and unwashed counts (in counts per minute, cpm) are scintillation measurements from the TCA-washed and the unwashed papers, respectively. For the sfGFP reactions, [leucine] was equal to 2,005 μM, and the molecular weight of the product was 26,860. There are 18 leucines in sfGFP, and the number 1,000 in the denominator was included so that the final [sfGFP] is in μg/ml. Although we provide the detailed procedure here, we do not expect that this procedure will need to be repeated for regenerating the color gradient.

The Laboratory Course

Student Demographics and Participation

In implementing this horizon-broadening experience, we chose a student population that was diverse in its demographic composition. Sequoia High School is a nationally acclaimed high school in Redwood City, CA, and was designated as a Title I Academic Achievement School and a California Distinguished School in 2007. Furthermore, the student population is very diverse; English is not the language spoken at home for the majority of the students. In the biotechnology class, there were a total of 90 students distributed in 4 sections. Of the 90 students, 43 were Title 1, 11 were English-learning, and 4 were Special Education students.

Campus Visit

Approximately 1 month before the laboratory course, we hosted the students from the Sequoia High School's biotechnology class at Stanford University. The field trip included a college-style lecture about CFPS and a 20-min laboratory tour. During the tour, the students were informed about different stages of protein production and analysis.

Laboratory Course

The teaching laboratory included four sessions: two 1.5-h laboratory periods flanked by two 50-min periods.

Session 1: (50 min)

In the first of these short periods, J.R.S. gave a half-hour lecture, in which he explained the CFPS platform and the role of each of the four variables (namely amino acids, cell extract, DNA, and phosphoenolpyruvate (PEP) concentration) in protein production. In the explanation, he likened protein production to building a house, where the amino acids (AAs) are the bricks or building blocks, the cell extract (which contains the catalysts) comprises the workers, the DNA contains the blueprint, and the PEP provides the money in the currency of ATP or GTP. We then showed a diagram depicting protein synthesis and asked the students to find a partner and, in groups of two, to identify the three enzymatic processes where these biological currencies are used. Two of these processes, translation and NTP generation for transcription, were explicitly shown on the CFPS diagram, while the third, charging the transfer RNAs (tRNAs) with amino acids, was less obvious. Therefore, by the end of the lecture, we had explained transcription and translation, including tRNA charging and ATP usage, at a molecular level.

After the lecture, K.C.J. divided the students into groups of 3–5 students each. Each group was assigned one of the four variables and was asked to evaluate the effect of four different concentrations of the investigated component on the production rate and final yield of super-folder green fluorescent protein (sfGFP). For example, the groups investigating the energy source, PEP, were asked to assess sfGFP production when the CFPS reactions initially contained 20, 30, 40, or 50 mM PEP (the published standard is 33 mM). We gave them a 200 mM stock solution of PEP and asked them to serially dilute this solution down to 160, 120, and 80 mM PEP. These solutions would then be diluted fourfold upon addition to the CFPS reaction mixtures to achieve the desired concentrations in the CFPS reactions. To help with the serial dilutions, we provided them with a partially completed table, in which they were asked to write the amount of PEP stock solution and water required for each dilution (Fig. 1a). Groups that completed their dilution tables before the end of the period started to prepare their stock solutions, but the majority of the groups were only able to complete their dilution tables.

The DNA and AA groups followed the same protocol as the PEP groups. Because cell extract loses activity after repeated freeze-thaws, the cell extract groups did not carry out serial dilutions to prepare stock solutions at different concentrations. Instead, they completed a slightly different dilution table (Fig. 1b), which was intended for use on the day of the actual experiment (Session 3).

Session 2: (90 min)

In session 1, some of the DNA, AA, and PEP groups had difficulty completing their dilution tables. Therefore, at the beginning of the class, K.C.J. went over the concept of serial dilutions with the students and used the following formula in the explanation:

Tables given to students

A **PEP Dilution Table:**

Bulk stock concentration	200 mM	160 mM	120 mM
Bulk stock volume to be added			
Water to be added			
Final volume	100 µL	100 µL	90 µL
Final concentration of 4X stock solution	160 mM	120 mM	80 mM
Concentration in the CFPS reaction	40 mM	30 mM	20 mM

AA Dilution Table:

Bulk stock concentration	16 mM	8 mM	4 mM
Bulk stock volume to be added			
Water to be added			
Final volume	50 µL	50 µL	50 µL
Final concentration of 4X stock solution	8 mM	4 mM	2 mM
Concentration in the CFPS reaction	2 mM	1 mM	0.5 mM

DNA Dilution Table:

Bulk stock concentration	48 nM	36 nM	24 nM
Bulk stock volume to be added			
Water to be added			
Final volume	48 µL	45 µL	40 µL
Final concentration of 4X stock solution	36 nM	24 nM	12 nM
Concentration in the CFPS reaction	9 nM	6 nM	3 nM

B **Cell Extract Dilution Table:**

Final concentration:	15% v/v	20% v/v	25% v/v	30% v/v
2X premix				
cell extract				
water				
Final Volume	60 µl	60 µl	60 µl	60 µl

Completed tables

PEP Dilution Table:

Bulk stock concentration	200 mM	160 mM	120 mM
Bulk stock volume to be added	80 µL	75 µL	60 µL
Water to be added	20 µL	25 µL	30 µL
Final volume	100 µL	100 µL	90 µL
Final concentration of 4X stock solution	160 mM	120 mM	80 mM
Concentration in the CFPS reaction	40 mM	30 mM	20 mM

AA Dilution Table:

Bulk stock concentration	16 mM	8 mM	4 mM
Bulk stock volume to be added	25 µL	25 µL	25 µL
Water to be added	25 µL	25 µL	25 µL
Final volume	50 µL	50 µL	50 µL
Final concentration of 4X stock solution	8 mM	4 mM	2 mM
Concentration in the CFPS reaction	2 mM	1 mM	0.5 mM

DNA Dilution Table:

Bulk stock concentration	48 nM	36 nM	24 nM
Bulk stock volume to be added	36 µL	30 µL	20 µL
Water to be added	12 µL	15 µL	20 µL
Final volume	48 µL	45 µL	40 µL
Final concentration of 4X stock solution	36 nM	24 nM	12 nM
Concentration in the CFPS reaction	9 nM	6 nM	3 nM

Cell Extract Dilution Table:

Final concentration:	15% v/v	20% v/v	25% v/v	30% v/v
2X premix	30 µl	30 µl	30 µl	30 µl
cell extract	9 µl	12 µl	15 µl	18 µl
water	21 µl	18 µl	15 µl	12 µl
Final Volume	60 µl	60 µl	60 µl	60 µl

Fig. 1 Dilution tables for the PEP, AA, DNA (**a**) and cell extract (**b**) groups. The students filled out these tables before diluting their stock solutions

$$\frac{\text{New concentration}}{\text{Old concentration}} = \frac{\text{Added volume}}{\text{Final volume}} \quad (2)$$

Each successive solution was used as the stock for the next dilution. For example, the PEP groups used the 200 mM solution as the stock for preparing the 160 mM solution, and the new 160 mM solution as the stock for the 120 mM solution, and so on (Fig. 1a). All of the DNA, AA, and PEP groups completed the preparation of their different stock solutions, which were then stored at 4 °C until session 3. After the stock solutions were prepared, C.A. explained to the students in detail how they would conduct their experiments. The students then wrote a detailed protocol, outlining each step of the experimental setup. The protocol for the cell extract groups (Table 1b) was slightly different than the others (Table 1a), since the cell extract loses activity after freeze–thaws.

Session 3: (90 min)

The sfGFP production experiment was conducted in this 1.5-h laboratory period. Following the protocols written in session 2, the groups quickly prepared their reaction solutions to ensure they would have observable protein production within the single class period. After the students

Table 1 Experimental protocol for the PEP, AA, DNA (A), and cell extract (B) groups

(A) Experimental protocol for the PEP, AA, and DNA groups

The final volume of each reaction mixture is 20 µl

The cell extract concentration in the final reaction is 25 % v/v

The 2× premix solution contains all of the ingredients at twice the final concentration except the cell extract

1. Label 8 microcentrifuge tubes with the reagent concentrations you are testing. Since there are 2 tubes at each concentration, use the letters A and B to distinguish the tubes. Set these aside for now
2. Make a master mix by combining 100 µl of 2× premix and 50 µl of cell extract; this is sufficient for a total of 8 reactions plus an excess to avoid running out of solution during its distribution. Mix well by pipetting up and down
3. Distribute 15 µl of this master mix to each of the 8 reaction tubes
4. In each tube, add 5 µl of the appropriate 4× PEP, AA or DNA stock solution to obtain a final volume of 20 µl
5. Mix the final reactions well by pipetting up and down several times
6. Start incubating the reactions in the 37 °C water bath. Start your timer as soon as all of the tubes are in
7. Every 15 min, take your tubes out and estimate the sfGFP concentrations in each tube using the color gradient. Record the concentration that best matches your tube in a table. Try to make your measurements quickly so that the tubes are outside the 37 °C water bath as briefly as possible

(*B) Experimental protocol for the cell extract groups*

The final volume of each reaction mixture is 20 µl

The 2× premix solution contains all of the ingredients at twice the final concentration except the cell extract

1. Label 8 microcentrifuge tubes with the cell extract concentrations you are testing. Since there are 2 tubes at each concentration, use the letters A and B to distinguish the tubes. Set these aside for now
2. Label an additional 4 tubes with the cell extract concentrations. Using the dilution table that you completed, combine the 2× premix, cell extract, and water; this is sufficient for a total of 2 reactions plus an excess to avoid running out of solution during its distribution. Mix well by pipetting up and down several times
3. Distribute 20 µl of each of the 4 solutions to 2 labeled tubes for each cell extract concentration
4. Mix the final reactions well by pipetting up and down several times
5. Start incubating the reactions in the 37 °C water bath. Start your timer as soon as all of the tubes are in
6. Every 15 min, take your tubes out and estimate the sfGFP concentrations in each tube using the color gradient. Record the concentration that best matches your tube in a table. Try to make your measurements quickly so that the tubes are outside the 37 °C water bath as briefly as possible

The cell extract groups followed a different protocol because repeated freeze-thaws reduce the extract quality. Students wrote these protocols on the day prior to the experiment (during Session 2) after the experimental setup was explained in detail

started incubating the reactions in a 37 °C water bath, a sfGFP color gradient (Fig. 2) was distributed for estimating the product concentrations. Each group was also provided with a "blank" CFPS reaction solution that contained all of the reagents except DNA and thus did not produce any sfGFP. This enabled a direct comparison so that the students could better evaluate product accumulation. Because of the yellowish hue of the initial cell-free solutions, it can otherwise be difficult to distinguish the green color caused by the sfGFP at the earlier time points. The groups recorded their estimates of sfGFP concentration every 15 min until the end of class. These data were then collected by K.C.J. and sent to C.A.

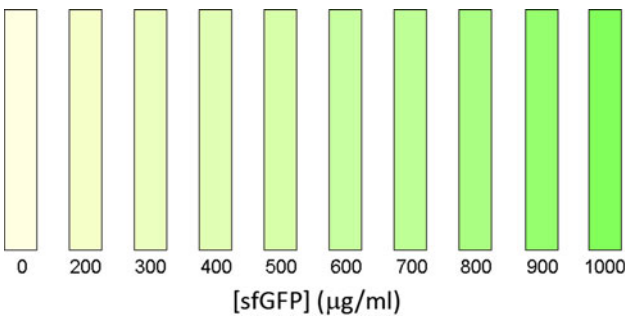

Fig. 2 Color chart used by the students to estimate sfGFP concentrations

Session 4: (50 min)

Before the lecture, C.A. compiled all of the data produced by the student groups and prepared two sets of graphs for each variable. In one set, the data obtained by the students in that particular section were graphed. These were compared to the second set, which contained the data for that variable from all of the groups in the 4 sections.

At the beginning of the lecture, students in the same group sat next to one another, and the groups investigating the same variable were also seated together. J.R.S. reviewed ATP usage and the importance of each variable (DNA, AA, PEP, and cell extract) in protein synthesis. He discussed the data for each variable and summarized the main results of the experiment. Afterward, we asked the

students to find a partner and think about how the procedure could be modified to obtain more products in light of these results. Finally, we encouraged the students to think outside the box and suggest other parameters they could investigate to increase sfGFP production.

Additional Considerations

In total, this laboratory experience used 4 h and 40 min of class time, which is a substantial time commitment. If less time is available, the serial dilution step (Session 2) can be omitted. Instead, students can be provided with a master stock solution for each component, and they can carry out the necessary dilutions on the day of the experiment (Session 3) just before beginning their CFPS reactions (similar to the cell extract groups). Alternatively, the teacher can prepare the different dilutions of the variable component solutions ahead of time.

The standard curve in Fig. 5 was generated by us in the laboratory and does not need to be repeated. This curve should provide a good foundation for future experiments. In addition, it is important that the components of the CFPS solution be well-mixed for the reactions to be productive. Thus, it is imperative for the students to have practiced micropipetting; in fact, this laboratory would provide a good opportunity for the teacher to test their micropipetting skills. Finally, the teacher should be aware that the cell extract contains a small number of unlysed cells. Therefore, if the cell-free reactions are incubated for greater than 10 or 12 h at elevated temperatures (30 or 37 °C), these cells may grow and interfere with sfGFP synthesis and fluorescence.

Results

Twenty-six of the 30 student groups in the biotechnology class observed sfGFP production over the 75-min period. One group overestimated the concentrations of sfGFP in their tubes, while the reactions of three groups did not work as well as expected. Details are given below.

Cell-Free Synthesis of sfGFP

Variation in three of the four variables resulted in a significant difference in observed sfGFP accumulation over 60 min. Increasing the DNA concentration from 3 to 12 nM resulted in ~25 % higher sfGFP concentrations (Fig. 3a). Similar increases in sfGFP yield were observed when cell extract and PEP concentrations were increased from 15 to 30 % v/v, and from 20 to 50 mM, respectively (Fig. 3b, c). (In the case of PEP, the Mg^{2+} concentration was also increased from 12.5 to 31.25 mM, since 125 mM Mg^{2+} was included in the 200 mM PEP stock solution.) However, increasing the concentration of amino acids from 0.5 to 2 mM did not significantly affect sfGFP production after 60 min (Fig. 3d). The large error bars were a result of having a limited number of replicates and assay error, since the students cannot accurately determine the differences in color when the product concentrations

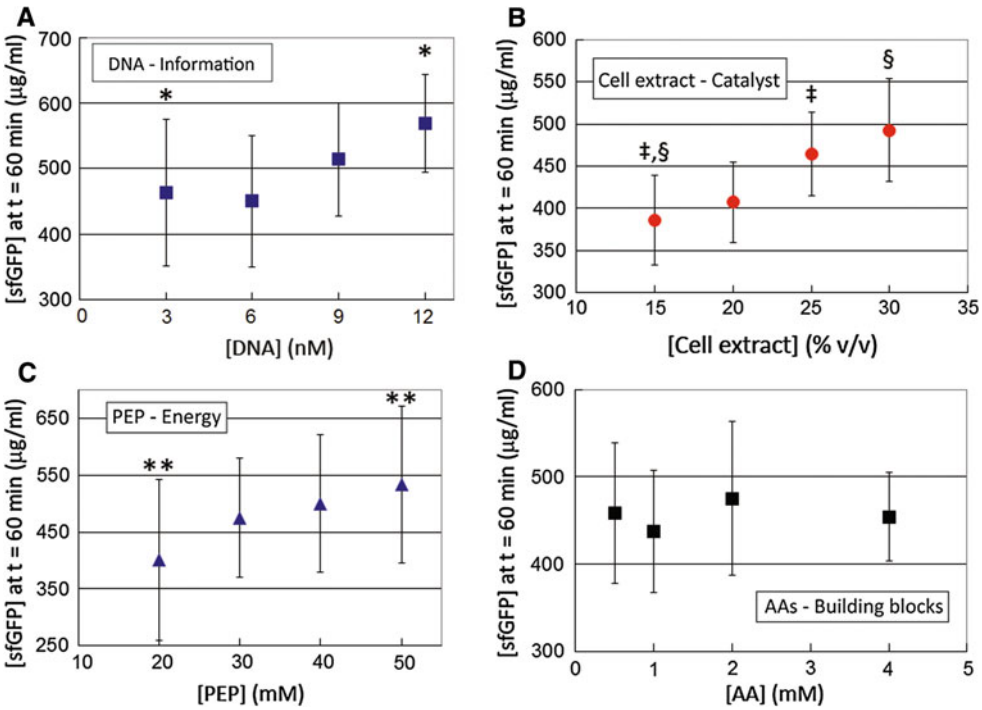

Fig. 3 sfGFP accumulation after 60 min at different DNA (**a**), cell extract (**b**), PEP (**c**) and AA (**d**) concentrations. sfGFP estimates are the averages from the groups that correctly prepared their reactions. *Error bars* indicate ± 1 SD for 11–14 reactions. The p values for the different data sets are: $^{*}p = 0.016$, $^{\ddagger}p < 0.0001$, $^{\S}p < 0.001$, $^{**}p = 0.028$

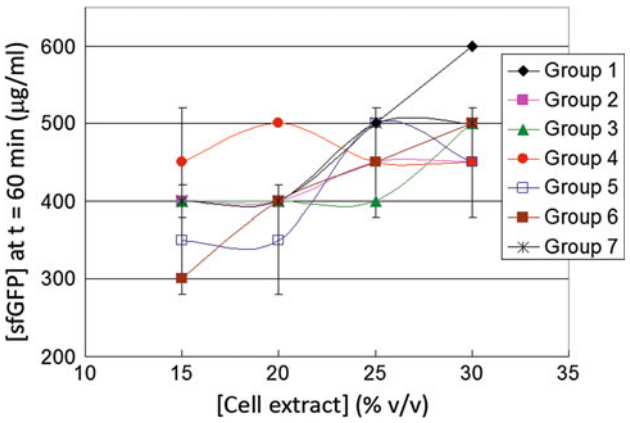

Fig. 4 sfGFP accumulation recorded by the individual groups after 60 min at different cell extract concentrations. *Each point* represents an average of duplicate reactions. Similar trends were obtained with DNA and PEP groups

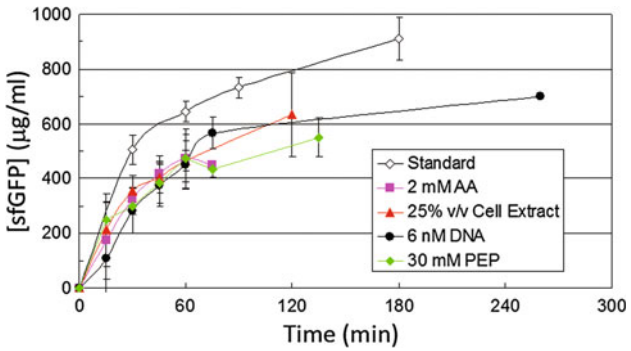

Fig. 5 Comparison of the yields obtained from the students' reactions to the laboratory standard ("Standard"). The students' values were obtained by estimating [sfGFP] from the *color* gradient, while the laboratory values were calculated from radioactivity measurements

are within 100 µg/ml of one another (Fig. 4). However, when all of the students' data were combined, the differences in sfGFP yields between the lowest and the highest DNA, cell extract, and PEP concentrations were statistically significant ($p < 0.03$). The observed trends were thus adequate for serving as a basis for class discussions.

When designing the experiment, we made sure that one of the four concentrations tested for each variable was the same as for the standard procedure. After the experiment, we compared the data obtained at these concentrations, namely 6 nM DNA, 2 mM AA, 25 % v/v cell extract, and 30 mM PEP, to the yields from standard reactions conducted in the Swartz laboratory (Fig. 5). Despite the crude product assessment method, the students' values for the sfGFP concentrations were within 50 % of those obtained using radioactive leucine incorporation. Furthermore, the students correctly observed the product accumulation curve characteristics of the PANOx SP cell-free system, where approximately half of the total protein is produced in the first 30 min at 37 °C. In the 90-min period, about half of the groups initiated their reactions within 15 min and thus were able to take 5 sets of measurements (up to 75 min), while the other half took longer and acquired only 4 sets of measurements (up to 60 min). To obtain more data, K.C.J. offered extra credit to students who would come at their next opportunity to take an additional time point. Several groups took advantage of this offer, and their data, taken at different time points, are also included in Fig. 5.

Of the 30 groups, only 4 produced data sets that were significantly different from those of the other groups conducting the same experiment. After class discussion, we were able to determine why their results differed. One of the AA groups had overestimated the sfGFP concentration because they used ultraviolet (UV) light rather than ambient light while estimating sfGFP concentrations (Fig. 6). We had encouraged the students to use the available UV lights only if they had trouble in distinguishing their reactions from the blank CFPS solution at early time points. Instead, this group used the black light for all of their measurements. One of the PEP groups observed almost no sfGFP production over 75 min. It turned out that they had added water instead of the PEP stock solution when formulating their final reactions. Finally, one AA group and one DNA group did not mix their reactions well and thus accumulated less sfGFP than others over 60 and 135 min, respectively. In the overall

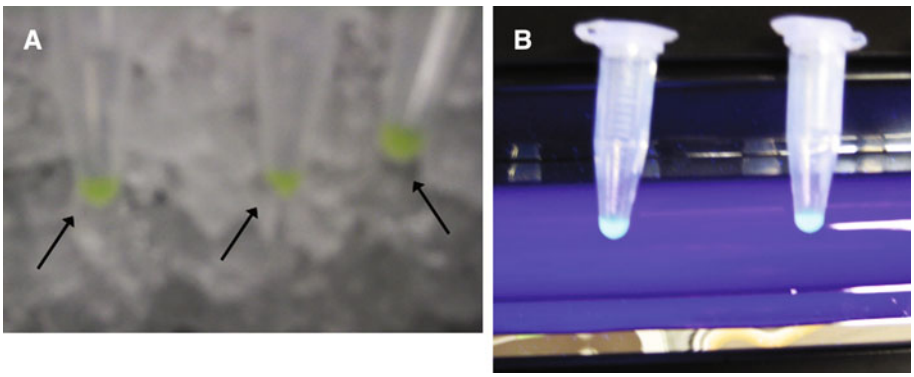

Fig. 6 CFPS reaction solutions containing sfGFP under ambient (**a**) and ultraviolet (**b**) light. In **a**, the *arrows* point to the 20 µl solution volumes

data analysis, we excluded the data from these groups from the "good" data set, and we compared the averages from the "good" data to the averages from the entire data set (see Supplementary Material) in the final class discussion.

Class Discussion

After discussing and summarizing the effects of the different variables on sfGFP accumulation, we asked the students to find a partner and to discuss what changes to the CFPS reaction conditions might further increase sfGFP production. We asked them to imagine themselves in a biotechnology company in charge of producing sfGFP and to suggest what changes they would make, based on their experimental data, to the CFPS system in order to obtain more protein (i.e., greener tubes). The students suggested increasing DNA, PEP, or cell extract concentrations or implementing these changes simultaneously. The latter answer allowed us to discuss the concept of synergy, through which making two or more simultaneous changes may result in a greater benefit than the sum of the benefits obtained from each of those changes. On the other hand, since increasing the AA concentration from 0.5 to 2 mM did not make a difference, the 0.5 mM concentration would be retained to avoid increasing the cost of sfGFP production.

We then asked the students to think outside the box and suggest other variables that may affect sfGFP production. Students responded that pH and temperature could be optimized, and additional reagents could be added during the course of the reaction (or a combination of these measures could be implemented) in future experimentation. Increasing the temperature from 37 to 40 or 41 °C may yield more sfGFP since reactions proceed faster at elevated temperatures. However, decreasing the temperature may also help in obtaining more folded and active protein despite reducing reaction rates. In explaining the effects of repeated reagent additions as a variable, we told the students about different types of processes (batch and fed-batch) in protein production. In a fed-batch process, the time and rate of reagent addition are common variables that are considered in process optimization.

During the discussion, the students asked two questions: (1) Why is the messenger RNA being continually made and degraded, thereby seemingly wasting energy? and (2) In CFPS, how do we control which proteins are made? To answer these questions, we discussed how genes are controlled via promoters and operators and how the balance between mRNA synthesis and degradation rates is a part of the mechanism to control protein concentrations. Furthermore, in CFPS, because the T7 promoter is directing the transcription of our gene of interest and because only the T7 RNA polymerase (that we add to the reaction) is producing mRNA, our protein of interest is the only product synthesized in the system.

Discussion

Overall, the experiments were very successful; the majority of the students were able to replicate the CFPS protocol used in the research laboratory and observe sfGFP accumulation over 60 min at rates similar to that achieved by full-time researchers. Furthermore, rather than simply completing a well-defined protocol with routine results, the students completed all of the steps of a research project: they tested hypotheses and, then guided by the results they obtained, were able to suggest future experiments to refine their hypothesis or to explore related directions. The hypotheses were that augmenting concentrations of DNA, AA, cell extract, and PEP (the energy source) would increase protein accumulation, since these variables are directly involved in transcription and translation. The students then used a previously unproven technique, namely the estimation of protein accumulation simply by comparing the color of their reaction tubes to a printed color gradient, to test their hypotheses. As in a true research project, some of the variables (DNA, extract, and PEP concentrations) in the tested range affected sfGFP accumulation, while one of them (AA) did not. Based on the results, the students were then able to suggest improvements to the protocol such as increasing concentrations of the investigated variables, in order to increase sfGFP accumulation. As the class discussion progressed, they went even further and suggested investigation of other variables for further optimization, such as pH, temperature, and using repeated reagent additions.

On the technical side, the students learned how to set up CFPS reactions, how to make serial dilutions of a stock solution, and how to estimate sfGFP concentrations by using a color chart. Their pipetting skills were also put to the test, since the CFPS reactions do not work as well if the reagents are not accurately dispensed and then well-mixed, as observed by two of the 30 groups. On the didactic side, two different sets of goals were accomplished. On the one hand, concepts related to biological processes such as central metabolism and transcription/translation were reinforced, while more general concepts such as the importance of temperature, reagent concentrations, and pH on chemical reactions were revisited. Through the class discussions, more advanced topics such as different kinds of bioprocesses (batch versus fed-batch) and gene control (promoters, operators and mRNA attenuation) were introduced. On the other hand, while it was not originally intended, the students also learned about problems related to conducting research: What would you do when you have

multiple sets of data for the same parameters? How do you distinguish the good sets from the bad ones? How would you use the results you obtained from one experiment to guide your future experiments? Given what you know about chemical reactions, what variables other than the ones you considered may you want to investigate to further improve your results? These are questions that full-time researchers tackle on a regular basis and that the high school students had a chance to consider as part of this laboratory experience.

This laboratory exercise can also be utilized in a college-level biology laboratory course. The course would reinforce the students' understanding of central metabolism, and protein synthesis, while also allowing the instructor to test their micropipetting skills. In the class discussions, different biological processes and research questions (such as the ones explained in the previous paragraph) can be examined. If a fluorimeter is available in the college teaching laboratory, visual estimates of sfGFP concentration from the color gradient can be compared to or replaced with fluorescence measurements.

Including the field trip and the class discussions, this laboratory experience consumed 5 periods. While this represents a significant time commitment, the experience achieves several teaching objectives. First, the laboratory course amounts to a full State Standard in genetics (California Department of Education 2000) and addresses a number of topics outlined in the National Science Education Standards (National Research Council 1996). It effectively reinforces concepts of the central dogma, recombinant DNA technology, and protein synthesis. Secondly, unlike many of the other high school laboratory experiments, in which students merely carry out well-established protocols such as gene amplification via PCR, this course involves true variable testing. High school students evaluate the effect on protein accumulation of four different variables that are directly involved in transcription and translation. In the process, they generate meaningful data using an easy and practical method. We believe this method of protein estimation provides the perfect compromise between practicality and accuracy, since more quantitative methods such as immunoassays or fluorimetry are also more laborious and expensive. If more significant differences within the investigated variables are desired, the experiments can be conducted for longer periods or the range of values for each variable can be increased. Finally, this laboratory course provides the high school students with a glimpse into the research experience. In the course, they test a hypothesis, obtain results, and then think through subsequent experiments that could further improve their results.

We also believe that this exercise is a great example of inquiry-based learning, in that: students work in groups to tackle different hypotheses, think through the experiments they are expected to conduct, and propose further experiments based on the results they obtain (Anderson 2002). It has been shown that an inquiry-based approach augments learning (Scruggs et al. 1993) and increases the students' motivation (Heywood and Heywood 1992) and their achievement (Basaga and NEED 1994). Furthermore, the combination of an in-class, hypothesis-driven laboratory exercise with an out-of-class visit of research facilities and interaction with full-time researchers could amount to a transformative experience for many of the students (Pugh et al. 2009). The overarching goal of our exercise was to instill a sense of personal attainability, such that these students would be motivated to pursue technical careers.

In this initial implementation of the laboratory exercise, we did not have the chance to formally evaluate its contribution to students' understanding of protein synthesis. This evaluation can be done in the form of a quiz or questionnaire. It may include questions such as: (1) How does each variable contribute to protein synthesis? (2) What are the important steps in protein synthesis? (3) In light of your results, what changes can be made to improve protein accumulation in CFPS? (4) What parameters (other than the ones investigated in this exercise) can be considered for further CFPS optimization? This exercise using CFPS will allow the students to understand protein synthesis as a series of chemical reactions rather than a biological black box, thereby enabling them to think about it on a more fundamental level.

In the case where there is no nearby research university, interaction with full-time researchers may not be possible, and the materials, the cell extract in particular, would have to be provided from another source. We do not believe the absence of a field trip will significantly diminish the pedagogical value of the laboratory experience. We also recently published a simplified protocol for reagent preparation, so that the source strain for the cell extract can be grown in shake flasks and most of the reagents can be combined and stored in a single solution (Yang et al. 2012). Furthermore, commercially available cell-free translation kits from companies such as RiNA GmbH (Berlin, Germany) and Life Technologies (Carlsbad, CA) can be used for this laboratory exercise; this would eliminate the need for time-consuming reagent preparation. Finally, to overcome reagent preparation as a potential limitation and to promote widespread adoption of this course, we will also be working with the Bay Area Biotechnology Education Consortium (BABEC) and certain companies in seeking to make the reagents widely available.

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