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TITLE:

Preparing Protein Producing Synthetic Cells using Cell Free Bacterial Extracts, Liposomes and Emulsion Transfer

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SUMMARY:

This protocol describes the method, materials, equipment and steps for bottom-up preparation of RNA and protein producing synthetic cells. The inner aqueous compartment of the synthetic cells contained the S30 bacterial lysate encapsulated within a lipid bilayer (i.e., stable liposomes), using a water-in-oil emulsion transfer method.

ABSTRACT:

The bottom-up assembly approach for construction of synthetic cells is an effective tool for isolating and investigating cellular processes in a cell mimicking environment. Furthermore, the development of cell-free expression systems has demonstrated the ability to reconstitute the protein production, transcription and translation processes (DNA→RNA→protein) in a controlled manner, harnessing synthetic biology. Here we describe a protocol for preparing a cell-free expression system, including the production of a potent bacterial lysate and encapsulating this lysate inside cholesterol-rich lipid-based giant unilamellar vesicles (GUVs) (i.e., stable liposomes), to form synthetic cells. The protocol describes the methods for preparing the components of the synthetic cells including the production of active bacterial lysates, followed by a detailed step-by-step preparation of the synthetic cells based on a water-in-oil emulsion transfer method. These facilitate the production of millions of synthetic cells in a simple and affordable manner with a high versatility for producing different types of proteins. The obtained synthetic cells can be used to investigate protein/RNA production and activity in an isolated environment, in directed evolution, and also as a controlled drug delivery platform for on-demand production of therapeutic proteins inside the body.

INTRODUCTION:

Synthetic cells are artificial cell-like particles, mimicking one or multiple functions of a living cell, such as the ability to divide, form membrane interactions, and synthesize proteins based on a genetic code[1-3]. Synthetic cells that enclose cell-free protein synthesis (CFPS) systems possess high modularity due to their ability to produce various proteins and RNA sequences following alterations in the DNA template. Presenting an attractive alternative to the current approaches of protein production, CFPS systems are based on cell lysate, purified components, or synthetic components and include all the transcription and translation machinery required for protein synthesis such as ribosomes, RNA polymerase, amino acids and energy sources (e.g., 3-phosphoglycerate and adenine triphosphate)[4-9]. The encapsulation of a CFPS system inside lipid vesicles enables the simple and efficient production of proteins without depending on a living cell[10]. Moreover, this platform allows synthesis of peptides that may degrade inside natural cells, produce proteins that are toxic to living cells, and modify proteins with non-natural amino acids[11,12]. Synthetic cells have been used as a model for research purposes investigating the minimal cell components required to enable cellular life from an evolutionary perspective[1,13]. Synthetic cells have also been used to build and implement genetic circuit and as models for directed evolution[14-16]. Other studies have focused on the ability of synthetic cells to mimic the biological activity of natural cells, aiming to replace damaged natural cells, such as beta cells in patients with diabetes[17]. Furthermore, the ability of these CFPS encapsulating synthetic cells to produce a variety of therapeutic proteins illustrates its potential to be incorporated into clinical use[18].

Here we describe a bottom-up lab-scale protocol (**Figure 1**) for the production of RNA and protein-producing synthetic cells based on a CFPS system encapsulated in a lipid vesicle. This shows the potential use of synthetic cell platforms as novel drug delivery systems for the onsite production of a therapeutic protein drug in vivo[19]. Previous studies have investigated the optimization of the CFPS reaction and the cell lysate preparation processes[4,8,20]. Moreover, several techniques have been applied for cell-sized liposome preparation, such as microfluidic and polymer-based droplet stabilization methods[21-23], which also differ in the liposomes' lipid composition[24-26]. In the presented protocol, synthetic cells are produced using a water-in-oil emulsion transfer method and the encapsulation process is carried out at low temperatures (<4

°C)[5,10,24,27,28]. These mild conditions have been found to be favorable for retaining the bio-functional integrity of the molecular machinery, namely ribosomes and proteins[27,29,30]. The lipid composition of the particles consists of both cholesterol and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). The first is found in all mammalian cell membranes and is essential for the stability, rigidity and permeability reduction of the membrane, and the latter mimics mammalian phospholipid composition[11,13]. The cellular transcription and translation molecular machinery are extracted from the BL21 (DE3) Escherichia coli (E. coli) strain, which is transformed with pAR1219 plasmid overexpressing T7 RNA polymerase to increase CFPS potency and protein synthesis. This system has been used to produce diagnostic and therapeutic proteins, with molecular weights of up to 66 kDa in vitro and in vivo[19,31]. The following protocol provides a simple and effective method for the production of the synthetic cell system, which can address a wide range of fundamental questions associated with protein synthesis in nature and can also be utilized for drug delivery applications.

PROTOCOL:

NOTE: Illustration of the complete synthetic cells' production protocol is presented in **Figure 1**. According to the user's needs, the protein expression (section 3.2) and synthetic cell formation (section 4) parts of the protocol can also be carried out independently (with some adaptations).

1. Preparation of S30-T7 lysate

1.1. Streak plate the *E. coli* BL21(DE3) bacteria transformed with the T7 RNA polymerase expressing pAR1219 plasmid on a LB-agar plate supplemented with 50 µg/mL ampicillin to obtain single colonies.

1.2. Prepare a starter solution: Inoculate a single colony into 5 mL of LB-media supplemented with 50 µg/mL ampicillin in a 100 mL Erlenmeyer flask and grow overnight using a floor incubator shaker at 250 rpm and 37 °C. Prepare duplicates.

1.3. Inoculate each 5 mL starter separately into 500 mL of TB media supplemented with 50 µg/mL ampicillin in a 2 L Erlenmeyer flask with baffles and grow it using a floor incubator shaker at 250 rpm and 37 °C until it reaches $OD_{600}\approx1$. Monitor periodically using a spectrophotometer.

1.4. Add 3 mL of 100 mM stock of IPTG (to reach 0.6 mM) for induction of T7 RNA polymerase expression and continue growing the culture until it reaches $OD_{600}\approx4$.

1.5. Transfer the solution from each Erlenmeyer flask into two 250 mL sterilized centrifuge tubes.

1.6. Centrifuge each at 7,000 x *g* for 10 min at 4 °C. Discard the supernatant.

NOTE: At this stage, the bacterial pellet can be stored at -20 °C for a few days before moving on to the next steps.

1.7. Re-suspend each pellet in 250 mL of cold (4 °C) S30 lysate buffer and centrifuge at 7,000 x *g* for 10 min at 4 °C.

NOTE: The S30 lysate buffer is used for maintaining protein stability after cell lysis is performed using the homogenizer in step 1.9. From this step forward, all steps until 1.12 should be carried out consecutively and rapidly.

1.8. Discard the supernatant and re-suspend all pellets together in 15 mL of cold S30 lysate buffer.

1.9. Homogenize at a working pressure of 15,000 psi, with an air pressure of 4 bar (two passes) for cell breakage. Avoid solution dilution for a more concentrated and active lysate.

1.10. Add 100 µL of 0.1 M DTT per 10 mL of the homogenized suspension.

1.11. Centrifuge the suspension at 24,700 x *g* for 30 min at 4 °C.

1.12. Perform the following step quickly for preserving the lysate activity: divide the supernatant one-by-one into 200 µL aliquots in precooled 1.5 mL vials and immediately snap freeze them with liquid nitrogen. Store at -80 °C for further use.

2. Preparation of lipids in oil solution

2.1. Dissolve POPC and cholesterol in chloroform (CAUTION) separately, each to a final concentration of 100 mg/mL. Vortex each vial separately.

2.1.1. Combine the components in a glass vial: add 50 µL of POPC, 50 µL of cholesterol and 500 µL of mineral oil. For 100 µL of inner solution, 2 vials of lipids in oil are required.

2.1.2. Vortex, and then heat for about 1 h at 80 °C in a chemical hood to evaporate the chloroform. Ensure that complete evaporation has occurred by following the specified time/conditions and monitoring the solution volume.

NOTE: The resulting lipid-in-oil solution can be stored at room temperature for up to two weeks. For improved results, it is recommended to use a fresh preparation before each experiment. Lipid and cholesterol ratios can be altered according to the desired membrane composition. A high concentration of cholesterol can lead to the formation of aggregates in the final synthetic cell solution.

CAUTION: Chloroform is classified as Irritant and Harmful and should therefore be treated with care and in areas with fume extraction.

3. Preparations of outer, inner and feeding solutions

3.1. Preparation of stock solutions

3.1.1. Prepare the stock solutions listed in **Table 2** using ultrapure water (UPW).

NOTE: Stock solutions should be prepared in advance and stored at -20 °C until further usage. Reagent 7 tends to form aggregates. Heating to 37 °C will reduce the aggregation. Slight aggregation will not affect the reaction significantly. Reagent 8 solution is milky and turbid.

3.2. Outer solution

3.2.1. Dissolve glucose in DNase/RNase-free H_2O to a final concentration of 200 mM.

3.2.2. For 100 µL of inner solution, prepare 1.6 mL of outer solution.

3.3. Inner solution

3.3.1. Add reagents 1-14 according to the amounts and concentration listed in **Table 2**. For example, for a final synthetic cell volume of 100 µL, prepare 100 µL of inner solution.

NOTE: UPW should be added to complete the final required volume. At this stage, the mixture can be stored at 4 °C for a few hours.

3.4. Feeding solution

3.4.1. Add all the reagents according to the amounts and concentration listed in **Table 3.**

NOTE: Use 1:1 ratio of feeding solution:inner solution. For a final synthetic cell volume of 100 µL, prepare 100 µL of feeding solution. It is recommended to prepare a small excess volume of outer, inner and feeding solution.

4. Preparation of synthetic cells

NOTE: The following volumes are adjusted for the preparation of 100 µL of synthetic cells.

4.1. Synthetic cells producing protein

4.1.1. In a 15 mL tube, place 12 mL of the outer solution and slowly add on top a layer of 500 µL of lipids in oil solution. Incubate at room temperature for 20 min.

4.1.2. Mix the inner solution ingredients on crushed ice to a final volume of 100 µL by thawing and adding S30-T7 Lysate (reagent 15) and DNA plasmid (reagent 16) to the stored mixture.

4.1.3. In another 1.5 mL tube, place 500 µL of lipids in oil solution and add 100 µL of the inner solution. Pipette up and down vigorously for 1 minute and vortex for another minute on level five.

4.1.3.1. Incubate for 10 min on crushed ice and slowly add the resulted emulsion on top of the oil phase in the 15 mL tube (from 4.1.1).

4.1.4. Centrifuge for 10 min at 100 x *g* and 4 °C and then centrifuge for 10 min at 400 x *g* and 4 °C. By the end of the centrifugation, a pellet at the bottom of the tube should be observed.

NOTE: Using a swinging bucket centrifuge rotor is preferred here for acquiring a better coverage of a second layer of lipids during the water-in-oil droplets' passage through the interphase. In case there is no observable pellet, centrifugation speed can be increased to 1000 x *g*. Otherwise, see the Discussion section referring to the specific gravity of the outer solution.

4.1.5. Extract the pellet.

4.1.5.1. Use a trimmed pipette tip loaded with approximately 400 µL of outer solution to extract the pellet. Release the outer solution while passing through the oil phase in order to collect only the pellet in the aqueous phase.

4.1.5.1.1. Wipe the tip after the extraction of the pellet to avoid transferring oil remains and transfer the pellet to a clean 1.5 mL tube.

4.1.6. Centrifuge for 10 min at 1,000 x *g* and 4 °C, remove the supernatant and re-suspend the pellet in 100 µL of feeding solution (1:1 ratio of inner:feeding solutions).

NOTE: A fixed angle centrifuge rotor may be used here as well.

4.1.7. For protein expression, incubate for 2 hours at 37 °C without shaking.

NOTE: Optimal incubation time varies between different proteins.

4.1.8. Evaluate the produced protein amount using a suitable method according to the target protein properties.

4.2. Recommended control groups

4.2.1. Inner solution and lysate activity confirmation

4.2.1.1. Prepare a complete inner solution (with DNA & S30-T7 lysate).

4.2.1.2. Immediately incubate the reaction above using a floor incubator shaker at 250 rpm or a thermomixer at 1200 rpm, at a constant temperature of 37 °C for 2 h.

NOTE: Adjust the incubation time to match the synthetic cells incubation time.

4.2.2. Synthetic cells

4.2.2.1. Negative control group: Prepare the protocol presented in 4.1 with inner solution without DNA.

4.2.2.2. Positive control: Prepare the protocol presented in 4.1 with inner solution containing a T7 plasmid encoding for a reporter gene.

NOTE: Add a positive control group comprised of a reporter gene, such as sfGFP, alongside the test groups to ensure the encapsulation efficiency step.

REPRESENTATIVE RESULTS:

We present a protocol for the preparation of synthetic cells by encapsulating a S30-T7 CFPS system based on BL21 *E. coli* inside lipid vesicles. A schematic description of the preparation process that includes an image of each stage is presented in **Figure 2.** The success of the synthetic cell preparation process is dependent on the appropriate performance of each stage and effected by different parameters. The protocol should be adjusted to accommodate the production of a specific protein.

Plasmids expressing the model protein super-folder Green Fluorescent Protein (sfGFP) and *Renilla* Luciferase under the T7 promoter were introduced into CFPS bulk reactions and synthetic cells, and protein production was evaluated using different methods, including western blot, flow cytometry, microscopy and spectroscopy (**Figure 3**). A verification of the sfGFP-His$_6$ tagged protein (~27 kDa[32]) production by western blot analysis is presented in **Figure 3A**. A sample of 30 µL of synthetic cells was mixed with 10 µL of a common SDS-PAGE sample buffer (containing the detergents sodium dodecyl sulfate (SDS) and β-mercaptoethanol) and boiled for 10 min (95 °C). We found that the combination of heat and detergents in the sample buffer is sufficient to disassemble the vesicles and to enable the running of proteins in the SDS-PAGE gel. Protein detection was performed using anti-His polyclonal primary antibody (diluted 1:12,000). As expected, while protein production is detected in samples containing sfGFP-encoding DNA templates ("+sfGFP DNA"), no protein is observed in negative control samples in which the DNA template was excluded ("-DNA"). This method can be applied to different protein types. According to Krinsky et al.[19], the sfGFP production yield obtained under the detailed protocol is 380 µg/mL in CFPS solution and 5.3 µg/mL, when the CFPS solution is encapsulated inside lipid vesicles.

When the protein that is produced inside the synthetic cells is fluorescent, its production can be evaluated using microscopy and flow cytometry-based methods. We analyzed the synthetic cells using a fluorescent microscope with a filter for GFP fluorescence (**Figure 3B**). Since the CFPS components have some autofluorescent, the image acquisition parameters should be adapted according to an appropriate negative control sample (synthetic cells with no DNA template, for example).

In addition, we used flow cytometry to determine the mean fluorescence intensity of sfGFP-producing synthetic cells, and the percentage of active synthetic cells, which can produce proteins within them (**Figure 3C I&II**). 10,000 events were collected for each analyzed sample. The synthetic cell production, which is described in this protocol, usually yields an active population of 21-25% within a solution with an approximated concentration of 10^7 synthetic cells/mL. The slight fluorescence intensity presented by “-DNA” samples in **Figure 3C II** is due to the autofluorescence of different components in the CFPS reaction such as the S30 lysate.

Representative size analysis based on the GFP signal (in diameter) of synthetic cells prepared by the method described above shows a mean value of 2.4±0.5 µm (**Figure 3D II**). As the formation of water in oil emulsion in this method is an outcome of applying mechanical forces, the size distribution of the particles might be affected by different factors, such as the method and speed of pipetting the emulsion up-and-down, the model of the vortex mixer machine, etc.

To test the versatility of synthetic cells' protein production, the expression of the reporter protein *Renilla* luciferase inside synthetic cells was analyzed (**Figure 3E**). The assay quantified *Renilla* luciferase activity by measuring the luminescence generated from the enzymatic reaction of luciferase and its substrate, h-coelenterazine. To induce the enzymatic reaction, a final concentration of 1 µM of h-coelenterazine was added to the synthetic cells (25 µL sample volume) just before the measurement. "-DNA" sample, not containing the luciferase-encoding DNA template was used as a negative control for testing luminescence due to non-enzymatic oxidation of the substrate. Luminescence was measured using a plate reader.

FIGURE AND TABLE LEGENDS:

Figure 1: An illustration of the process for typical synthetic cells preparation protocol. The process is divided into two steps. Step 1: Pre-experiment preparations including DNA plasmid purification, S30-T7 lysate preparation, stock solutions preparation required for the inner and feeding solutions, lipids-in-oil solution preparation and outer solution preparation. Step 2: Synthetic cell formation that includes feeding and inner solutions preparation, synthetic cells preparation and analysis. An example of the required volume of each ingredient for preparing 100 µL of synthetic cells solution is presented in blue in brackets.

Figure 2: Schematic illustration of the synthetic cell preparation protocol. An image of a well-performed process illustrates each stage of the protocol. This figure has been modified from Krinsky et al.[19].

Figure 3: **Representative results of the production of sfGFP and *Renilla* luciferase inside synthetic cells.** (**A**) A western blot analysis of sfGFP-His6 production in both CFPS bulk reactions and inside synthetic cells. Protein detection was carried out using anti-His polyclonal primary antibody (diluted 1:12,000). Purified sfGFP-His6 was used as a positive control (7.8 µg). Samples without DNA templates were used as a negative control for the production analysis ("-DNA"). (**B**) Representative images of sfGFP producing synthetic cells taken using a fluorescent microscope with a bright-field and a GFP filter. Scale bars = 50 µm. (**C**) I&II Flow cytometry analysis of sfGFP producing synthetic cells activity (Data collected using digital, 4-laser analyzer). Samples were measured after 3 h of incubation. 10,000 events were collected for each analyzed sample. FSC (500 V) and SSC (300 V) filters were used to define the total synthetic cell population. Then, a FITC (600 V) filter was used to detect GFP fluorescence.. (I) Calculation of the active synthetic cell population [%] was based on GFP fluorescence intensity threshold, defined by the "-DNA" sample (red histogram), allowing an error of ~1%. (II) Mean fluorescence intensity of sfGFP producing synthetic cells. This value was calculated from the active synthetic cell population and normalized to the "-DNA" sample by dividing the mean fluorescence of the active synthetic cells by the mean of the synthetic cells without sfGFP-DNA. (**D**) I&II Synthetic cell size analysis. The emission spectrum was detected by 505-560 nm and 642-745 nm for GFP and bright field signals, respectively. (I) A representative image of the analyzed synthetic cells. (II) Synthetic cells' size distribution. Analysis was performed and the diameter distributions of the active synthetic cells was calculated based on the GFP signal. (**E**) Production of active *Renilla* luciferase inside synthetic cells. Luciferase activity was quantified with luminescence measurements after the addition of 1 µM h-coelenterazine using a plate

reader and presented as light intensity [RLU] x 10^6.

Table 1: Buffer and stock solutions preparation.

Table 2: Inner solution composition.
*Add calculated DNA plasmid volume to obtain a final concentration of 10 µg/mL.
**Adjust to the required final volume by adding UPW.

Table 3: Feeding solution composition.

Table 4: Required solutions for synthetic cells preparation.

DISCUSSION:

This protocol introduces a simple and affordable method for the production of large quantities of protein-producing synthetic cells. The yield of active cells is dependent on careful and accurate execution of the protocol with emphasis on several critical steps. In the lysate preparation section of this method, it is essential to reach the appropriate bacteria density before cell lysis to achieve a sufficient amount of proteins in the bacterial lysate. Second, the lysis process should be performed at 4 °C and the lysate frozen quickly with liquid nitrogen to maintain protein activity. Moreover, in this protocol we used *E. coli* BL21(DE3) cells transformed with pAR1219 plasmid expressing T7 RNA polymerase. In cases where a different strain of bacteria is used, changes to the lysate concentration may be required to reach a satisfactory amount of RNA polymerase and ribosomes. Even when the same bacterial strain is used, different lysate productions may have some batch-to-batch variability.

The vesicle production section also includes a couple of significant steps. Solubilization of the lipids and removal of the chloroform from the mineral oil is important for establishing the water-in-oil emulsion. The centrifugation of the water-in-oil droplets into the outer aqueous buffer is also a key step in the protocol to generate synthetic cells with a lipid bilayer. Changes in the inner solution of the vesicles and the lipid composition might lead to a layer of droplets at the oil-water interphase without any observable pellet. To overcome this, a quick solution is to increase the centrifugation speed to 1,000 x *g* in the emulsion transfer step. In case this does not solve the issue, the specific gravity of the outer aqueous solution can be altered ensuring that the inner solution will have a higher specific gravity than the outer solution. Furthermore, the osmolality of the inner solution may also vary between different lysate sources and productions, ranging between 800-1100 mOsm/kg. Variability in the inner solution's osmolality is mostly due to changing concentrations of the lysate during its production process. Usually this does not lead to significant changes in protein production, yet a large dilution might lead to a reduced yield due to low concentrations of the transcription and translation enzymes in the lysate itself. Measuring the total protein concentration in each lysate batch can assist in tuning lysate concentrations in the inner solution to maintain constant values (approximately 22 mg/mL measured by Bradford assay).

Nevertheless, this method has some limitations that should be mentioned. Not all of the generated synthetic cells are active and capable of producing proteins due to incomplete encapsulation of all the required components. We measured approximately 21-25% of active cells when producing sfGFP expressing synthetic cells using flow cytometry (no significant size differences were observed between active and inactive cells). Oil and lipid excess residues occasionally remain in the bilayer lipid membrane after the transfer of the synthetic cells into the aqueous phase. Optimizing the lipid and cholesterol concentrations in the oil phase can improve this issue. It is also important to note that the size distribution of the obtained synthetic cells is quite wide in comparison to alternative microfluidic methods, with vesicles ranging from approximately 1-50 µm.

The high yield of the emulsion transfer method makes it especially suitable for synthetic cells encapsulating cell-free protein synthesis systems for therapeutic protein production. Different variations of the emulsion transfer method have been used in synthetic cell studies and included changes in the oil preparation procedure, membrane composition, sample volume, inner solution to oil ratio and centrifugation speeds[5,24,28]. Microfluidic and polymer-based droplet stabilization methods have also been used for synthetic cell preparation, however, encapsulation of the whole CFPS system has not been performed yet with these methods[21-23].

The synthetic cells obtained by this method were shown in vivo to produce proteins and treat cancer in murine models[19]. The capabilities of these cells can be further expanded in the future beyond protein expression. Integration of other cellular processes such as cellular communication, cytoskeleton modification and cell division in synthetic cells have been recently described[33-36]. Substitution of the bacterial lysate with eukaryotic cell lysate will allow expression of proteins with higher complexity and post-translational modifications, and will perhaps be less immunogenic even without a cleaning procedure, therefore opening more therapeutic frontiers[37].

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Blain, J. C., Szostak, J. W. Progress toward synthetic cells. *Annual review of biochemistry.* **83**, 615-640 (2014).

2 Richmond, D. L. et al. Forming giant vesicles with controlled membrane composition, asymmetry, and contents. *Proceedings of the National Academy of Sciences.* **108** (23), 9431-9436 (2011).

3 Deshpande, S., Spoelstra, W. K., van Doorn, M., Kerssemakers, J., Dekker, C. Mechanical division of cell-sized liposomes. *ACS Nano.* **12** (3), 2560-2568 (2018).

4 Liu, D. V., Zawada, J. F., Swartz, J. R. . Streamlining Escherichia coli S30 extract preparation for economical cell-free protein synthesis. *Biotechnology Progress.* **21** (2), 460-465 (2005).

5 Stano, P. Gene Expression Inside Liposomes: From Early Studies to Current Protocols. *Chemistry–A European Journal.* (2019).

6 Lewandowski, B. et al. Sequence-specific peptide synthesis by an artificial small-molecule machine. *Science.* **339** (6116), 189-193 (2013).

7 Li, J. et al. Cogenerating synthetic parts toward a self-replicating system. *ACS Synthetic Biology.* **6** (7), 1327-1336 (2017).

8 Kim, T. W. et al. An economical and highly productive cell-free protein synthesis system utilizing fructose-1,6-bisphosphate as an energy source. *Journal of Biotechnology.* **130** (4), 389-393 (2007).

9 Kim, T. W. et al. Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system. *Journal of Biotechnology.* **126** (4), 554-561 (2006).

10 Noireaux, V.,, Libchaber, A. A vesicle bioreactor as a step toward an artificial cell assembly. *Proceedings of the National Academy of Sciences.* **101** (51), 17669-17674 (2004).

11 He, M., He, Y., Luo, Q., Wang, M. From DNA to protein: No living cells required. *Process Biochemistry.* **46** (3), 615-620 (2011).

12 Casteleijn, M. G., Urtti, A., Sarkhel, S. Expression without boundaries: cell-free protein synthesis in pharmaceutical research. *International Journal of Pharmaceutics.* **440** (1), 39-47 (2013).

13 Luisi, P. L., Ferri, F., Stano, P. Approaches to semi-synthetic minimal cells: a review. *Naturwissenschaften.* **93** (1), 1-13 (2006).

14 Adamala, K. P., Martin-Alarcon, D. A., Guthrie-Honea, K. R., Boyden, E. S. Engineering genetic circuit interactions within and between synthetic minimal cells. *Nature Chemistry.* **9** (5), 431-439 (2017).

15 Ding, Y., Contreras-Llano, L. E., Morris, E., Mao, M., Tan, C. Minimizing Context Dependency of Gene Networks Using Artificial Cells. *ACS Applied Materials & Interfaces.* **10** (36), 30137-30146 (2018).

16 Arnold, F. H. Design by directed evolution. *Accounts of Chemical Research.* **31** (3), 125-131 (1998).

17 Chen, Z. et al. Synthetic beta cells for fusion-mediated dynamic insulin secretion. *Nature Chemical Biology.* **14** (1), 86-93 (2018).

18 Mohr, B. P., Retterer, S. T., Doktycz, M. J. While-you-wait proteins? Producing biomolecules at the point of need. *Expert Review of Proteomics.* **13** (8), 707-709 (2016).

19 Krinsky, N. et al. Synthetic Cells Synthesize Therapeutic Proteins inside Tumors. *Advanced Healthcare Materials.* **7** (9), e1701163 (2018).

20 Kim, T. W., Kim, D. M., Choi, C. Y. Rapid production of milligram quantities of proteins in a batch cell-free protein synthesis system. *Journal of Biotechnology.* **124** (2), 373-380 (2006).

21 Deng, N.-N., Yelleswarapu, M., Zheng, L., Huck, W. T. Microfluidic assembly of monodisperse vesosomes as artificial cell models. *Journal of the American Chemical Society.* **139** (2), 587-590 (2016).

22 Deshpande, S., Caspi, Y., Meijering, A. E., Dekker, C. Octanol-assisted liposome assembly on chip. *Nature Communications.* **7**, 10447 (2016).

23 Göpfrich, K. et al. One-Pot Assembly of Complex Giant Unilamellar Vesicle-Based Synthetic Cells. *ACS synthetic biology.* (2019).

24 Fujii, S. et al. Liposome display for in vitro selection and evolution of membrane proteins. *Nature Protocols.* **9** (7), 1578 (2014).

25 Periasamy, A. et al. Cell-free protein synthesis of membrane (1,3)-beta-d-glucan (curdlan) synthase: co-translational insertion in liposomes and reconstitution in nanodiscs. *Biochim Biophys Acta.* **1828** (2), 743-757 (2013).

26 Kalmbach, R. et al. Functional cell-free synthesis of a seven helix membrane protein: in situ insertion of bacteriorhodopsin into liposomes. *Journal of Molecular Biology.* **371** (3), 639-648 (2007).

27 Nishimura, K. et al. Cell-free protein synthesis inside giant unilamellar vesicles analyzed by flow cytometry. *Langmuir.* **28** (22), 8426-8432 (2012).

28 Rampioni, G. et al. Synthetic cells produce a quorum sensing chemical signal perceived by Pseudomonas aeruginosa. *Chemical Communications.* **54** (17), 2090-2093 (2018).

29 Stano, P., Kuruma, Y., de Souza, T. P., Luisi, P. L. *Biosynthesis of proteins inside liposomes*. 127-145 (Humana Press., 2010).

30 Pautot, S., Frisken, B. J., Weitz, D. A. Production of unilamellar vesicles using an inverted emulsion. *Langmuir.* **19** (7), 2870-2879 (2003).

31 Krinsky, N. et al. A Simple and Rapid Method for Preparing a Cell-Free Bacterial Lysate for Protein Synthesis. *PLoS One.* **11** (10), e0165137 (2016).

32 Pédelacq, J.-D., Cabantous, S., Tran, T., Terwilliger, T. C., Waldo, G. S. Engineering and characterization of a superfolder green fluorescent protein. *Nature Biotechnology.* **24** (1), 79 (2006).

33 Osawa, M., Erickson, H. P. Liposome division by a simple bacterial division machinery. *Proceedings of the National Academy of Sciences.* **110** (27), 11000-11004 (2013).

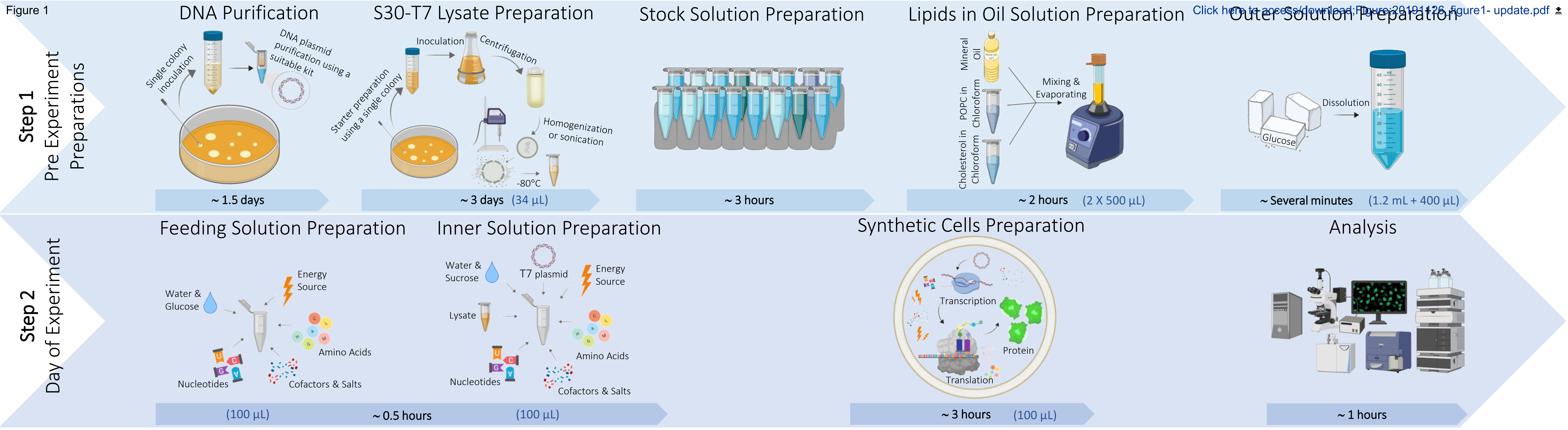
34 Merkle, D., Kahya, N., Schwille, P. Reconstitution and anchoring of cytoskeleton inside giant unilamellar vesicles. *ChemBioChem.* **9** (16), 2673-2681 (2008).

35 Vleugel, M., Roth, S., Groenendijk, C. F., Dogterom, M. Reconstitution of basic mitotic spindles in spherical emulsion droplets. *Journal of Visualized Experiments.* e54278 (2016).

36 Bayoumi, M., Bayley, H., Maglia, G., Sapra, K. T. Multi-compartment encapsulation of communicating droplets and droplet networks in hydrogel as a model for artificial cells. *Scientific Reports.* **7**, 45167 (2017).

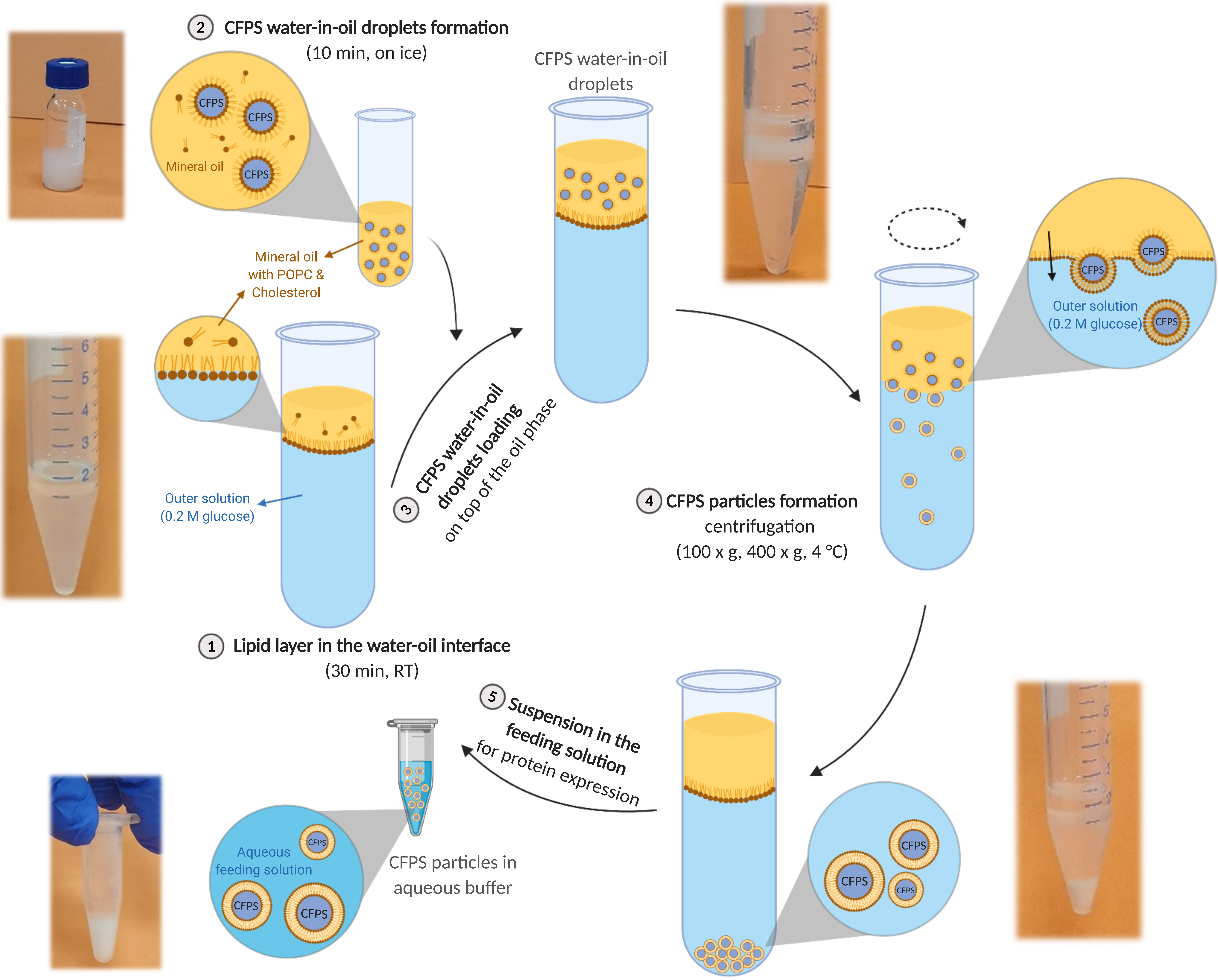
37 Carlson, E. D., Gan, R., Hodgman, C. E., Jewett, M. C. Cell-free protein synthesis: applications come of age. *Biotechnology Advances.* **30** (5), 1185-1194 (2012).

Figure 1



Step 1
Pre Experiment Preparations
DNA Purification
Single colony inoculation
DNA plasmid purification using a suitable kit
~ 1.5 days
S30-T7 Lysate Preparation
Starter preparation using a single colony
Inoculation
Centrifugation
Homogenization or sonication
-80°C
~ 3 days (34 µL)
Stock Solution Preparation
~ 3 hours
Lipids in Oil Solution Preparation
Mineral Oil
POPC in Chloroform
Cholesterol in Chloroform
Mixing & Evaporating
~ 2 hours (2 X 500 µL)
Outer Solution Preparation
Glucose
Dissolution
~ Several minutes (1.2 mL + 400 µL)
Step 2
Day of Experiment
Feeding Solution Preparation
Water & Glucose
Energy Source
Amino Acids
Nucleotides
Cofactors & Salts
(100 µL)
~ 0.5 hours
Inner Solution Preparation
Water & Sucrose
T7 plasmid
Energy Source
Lysate
Amino Acids
Nucleotides
Cofactors & Salts
(100 µL)
Synthetic Cells Preparation
Transcription
Protein
Translation
~ 3 hours (100 µL)
Analysis
~ 1 hours

Figure 2



2 CFPS water-in-oil droplets formation
(10 min, on ice)
CFPS
Mineral oil
Mineral oil with POPC & Cholesterol
Outer solution (0.2 M glucose)
1 Lipid layer in the water-oil interface
(30 min, RT)
CFPS water-in-oil droplets
3 CFPS water-in-oil droplets loading on top of the oil phase
4 CFPS particles formation
centrifugation
(100 x g, 400 x g, 4 °C)
Outer solution (0.2 M glucose)
5 Suspension in the feeding solution for protein expression
Aqueous feeding solution
CFPS particles in aqueous buffer

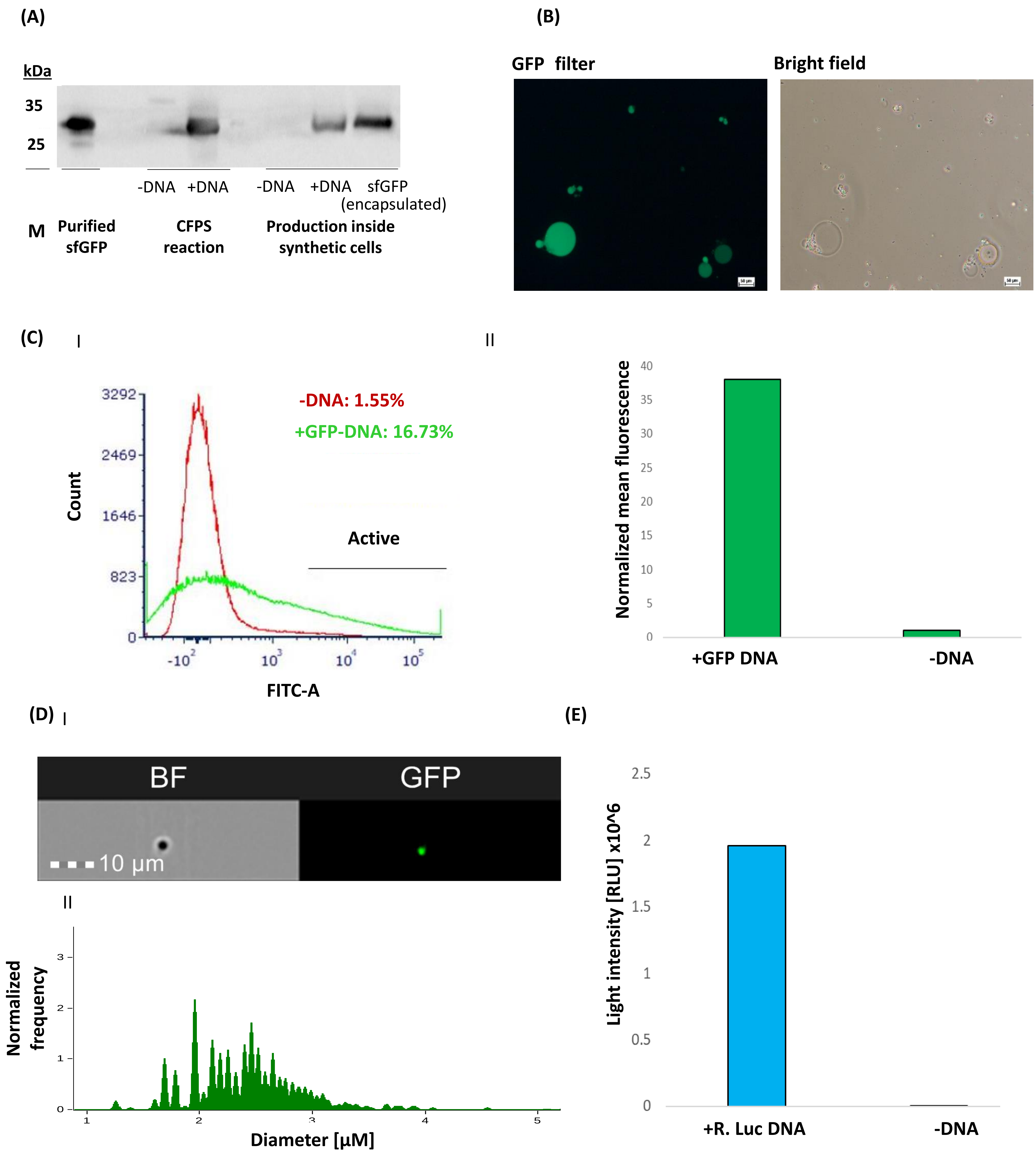

(A)
kDa
35
25
-DNA +DNA -DNA +DNA sfGFP (encapsulated)
M
Purified sfGFP
CFPS reaction
Production inside synthetic cells
(B)
GFP filter
Bright field
(C)
I
II
Count
3292
2469
1646
823
0
-DNA: 1.55%
+GFP-DNA: 16.73%
Active
FITC-A
Normalized mean fluorescence
40
35
30
25
20
15
10
5
0
+GFP DNA
-DNA
(D)
I
BF
GFP
10 µm
II
Normalized frequency
Diameter [µM]
(E)
Light intensity [RLU] x10^6
2.5
2
1.5
1
0.5
0
+R. Luc DNA
-DNA

Table 1

	Comments
Luria Bertani (LB) agar (1.5%) plate:	Prepare and sterilize. Add Ampicillin at a final concentration of 50 µg/mL only after cooling to room temperature.
10 g/L Bacto-tryptone	
10 g/L Sodium chloride (NaCl)	
5 g/L Bacto-Yeast extract	
15 g/L Agar agar purified	
50 µg/mL Ampicillin	
LB media (20 mL):	Minimal media. Prepare and sterilize in advance. Just prior to inoculating the bacteria, add Ampicillin at a final concentration of 50 µg/mL.
10 g/L Bacto-tryptone	
10 g/L Sodium chloride (NaCl)	
5 g/L Bacto-Yeast extract	
50 µg/mL Ampicillin	
Terrific Broth (TB) media (1 L):	Rich media. Prepare and sterilize in advance. Just prior to inoculating the bacteria, add Ampicillin at a final concentration of 50 µg/mL.
12 g/L Bacto-tryptone	
24 g/L Bacto-Yeast extract	
4% (v/v) Glycerol anhydrous	
2.32 g/L K2HPO4	
12.54 g/L KH2PO4	
50 µg/mL Ampicillin	
S30 lysate buffer (1.5 L):	Prepare and sterilize in advance (*excluding DTT and 2-mercaptoethanol). Store at -4 °C.
10 mM Tris-acetate at pH = 7.4	Trisma-base. Prepare and adjust pH to 7.4 using Acetic acid.
14 mM magnesium acetate	
60 mM potassium acetate	
*1 mM DTT	Add just prior to use
*0.5 mL/L 2-mercaptoethanol	Add just prior to use
1 M HEPES-KOH (pH = 8):	Dissolve HEPES to a final concentration of 1 M. Adjust to pH 8 using KOH solution.
HEPES	
Potassium hydroxide (KOH)	

Number	Reagent	Stock conc.	Final conc.	Final requested Inner solution volume					
				25 µL	50 µL	100 µL	200 µL	300 µL	400 µL
1	HEPES KOH pH=8	1 M	55 mM	1.375	2.75	5.5	11	16.5	22
2	Magnesium acetate	1 M	14 mM	0.35	0.7	1.4	2.8	4.2	5.6
3	Potassium acetate	1 M	50 mM	1.25	2.5	5	10	15	20
4	Ammonium acetate	5.2 M	155 mM	0.75	1.5	3	6	9	12
5	PEG 6000	50%	3%	1.5	3	6	12	18	24
6	3-PGA	0.5 M	40 mM	2	4	8	16	24	32
7	Amino acids - mixture I	50 M	2.5 mM	1.25	2.5	5	10	15	20
8	Amino acids - mixture II	50 M	2.5 mM	1.25	2.5	5	10	15	20
9	ATP	100 mM	1.2 mM	0.3	0.6	1.2	2.4	3.6	4.8
10	GTP	50 mM	1 mM	0.5	1	2	4	6	8
11	UTP	100 mM	0.8 mM	0.2	0.4	0.8	1.6	2.4	3.2
12	IPTG	100 mM	1 mM	0.25	0.5	1	2	3	4
13	Sucrose	2 M	200 mM	2.5	5	10	20	30	40
14 **	H2O UPW			**	**	**	**	**	**
15	S30-T7 Lysate		34%	8.5	17	34	68	102	136
16 *	DNA plasmid		10 µg/mL	*	*	*	*	*	*

Table 3

Number	Reagent	stock conc.	final conc.	Final requested feeding solution volume					
				25 µL	50 µL	100 µL	200 µL	300 µL	400 µL
1	HEPES KOH pH=8	1 M	83.3 mM	2.08	4.17	8.33	16.67	25	33.33
2	Magnesium acetate	1 M	21.2 mM	0.53	1.06	2.12	4.24	6.36	8.48
3	Potassium acetate	1 M	75.5 mM	1.89	3.78	7.55	15.1	22.66	30.2
4	Ammonium acetate	5.2 M	236.4 mM	1.14	2.27	4.55	9.09	13.64	18.18
5	PEG - 6000	50%	4.54%	2.27	4.54	9.08	18.16	27.24	36.32
6	3-PGA	0.5 M	60.1 mM	3	6.01	12.01	24.02	36.04	48.04
7	Amino acids - mixture I	50 mM	3.8 mM	1.89	3.78	7.56	15.12	22.68	30.24
8	Amino acids - mixture II	50 mM	3.8 mM	1.89	3.78	7.56	15.12	22.68	30.24
9	ATP	100 mM	1.8 mM	0.45	0.91	1.81	3.62	5.43	7.24
10	GTP	50 mM	1.5 mM	0.76	1.51	3.02	6.04	9.06	12.08
11	UTP	100 mM	1.2 mM	0.3	0.61	1.21	2.42	3.63	4.84
12	IPTG	100 mM	1.5 mM	0.38	0.76	1.51	3.02	4.53	6.04
13	Glucose	2 M	200 mM	2.5	5	10	20	30	40
14	H2O UPW			5.92	11.83	23.7	47.38	71.06	94.76

Solution & materials required	Comments
Lipids in oil	Store at room temperature until use. Prepared according to section 2
Outer solution	Prepared according to section 3.1
Inner solution	Prepared according to section 3.2. Prepare tests + controls samples according to section 4.1. Keep on crushed ice until use.
Feeding solution	Prepared according to section 3.3. Keep on crushed ice until use.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
A. Reagents required for step 1 (S30-T7 lysate preparation)			
E.coli BL21 (DE3)	NEB	C2527	E.coli BL21 (DE3).
pAR1219	Sigma	T2076	TargeTron vector for transformation.
Stock solution of 50 mg/mL Ampicillin	Sigma	A9518	Stored at -20 °C.
10 g/L Bacto-tryptone	BD Bioscience	211705	For preparation of Luria Bertani (LB) agar (1.5%) plate.
10 g/L Sodium chloride (NaCl)	Bio-Lab	19030591	
5 g/L Bacto-Yeast extract	BD Bioscience	212750	
15 g/L Agar agar purified	Merck	1.01614.5007	
50 µg/mL Ampicillin	Sigma	A9518	
10 g/L Bacto-tryptone	BD Bioscience	211705	For preparation of Luria Bertani (LB) media (20 mL).
10 g/L Sodium chloride (NaCl)	Bio-Lab	19030591	
5 g/L Bacto-Yeast extract	BD Bioscience	212750	
50 µg/mL Ampicillin	Sigma	A9518	
12 g/L Bacto-tryptone	BD Bioscience	211705	For preparation of Terrific Broth (TB) media (1 L).
24 g/L Bacto-Yeast extract	BD Bioscience	212750	
4% (v/v) Glycerol anhydrous	Bio-Lab	7120501	
2.32 g/L K2HPO4	Spectrum chemical	P1383	
12.54 g/L KH2PO4	Spectrum chemical	P1380	
50 µg/mL Ampicillin	Sigma	A9518	
Stock solution of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)	INALCO	INA-1758-1400	Filtered using 0.2 µm hydrophilic PVDF syringe filter.
Stock solution of 0.1 M dithiothreitol (DTT)	TCI	D1071	Filtered using 0.2 µm hydrophilic PVDF syringe filter.
10 mM Tris-acetate at pH = 7.4	Sigma	T1503	S30 lysate buffer (1.5 L)
14 mM magnesium acetate	Merck	1.05819.0250	
60 mM potassium acetate	Carlo Erba	470147	
1 mM DTT	TCI	D1071	
0.5 mL/L 2-mercaptoethanol	Sigma	M6250	

Equipment required for step 1			
100 mL sterilized Erlenmeyer flasks	Thermo Scientific	50-154-2846	2 flasks
2 L sterilized Erlenmeyer flasks with baffles	KIMAX-KIMBLE	25630	2 flasks
Floor incubator shaker	MRC	TOU-120-2	Laboratory shaker incubator 450x450mm, 400rpm, 70 °C
Centrifuge	Thermo Scientific	75004270	(75003340) - Fiberlite F10-6 x 100 LEX Fixed-Angle Rotor. Should enable at least 13,000 x g. * Pre-cooled to 4 °C.
High pressure homogenizer	AVESTIN	EmulsiFlex-C3	Pre-cooled to 4 °C.
-80^{o}C freezer	SO-LOW	U85-18	
Sterilized 1.5 mL plastic tubes	Eppendorf	30120086	Preferably pre-cooled to -20 °C.
Spectrophotometer	TECAN	IN-MNANO	Infinite M200 pro
96-well transparent plate	Thermo Scientific	167008	
Sterilized graduated cylinder	Corning		
Sterilized centrifuge tubes	Eppendorf	30120086	Preferably pre-cooled to -20 °C.
Sterilized pipette tips	Corning		Preferably pre-cooled to -20 °C.
Crushed ice bucket	Bel-Art	M18848-4001	
Small liquid nitrogen tank	NALGENE	4150-4000	
B. Reagents required for step 3 (lipids in oil solution preparation):			
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)	Lipoid	556400	Powder
Cholesterol	Sigma	C8667	Powder
Chloroform	Bio-Lab	3082301	
Mineral oil	Sigma	M5904	Light oil
Equipment required for step 2			
Vortex mixer	Scientific industries	SI-0256	
Heating block	TECHNE	FDB03AD	Pre-heated to 80 °C. Should enable controlled temperature.
2 mL screw neck glass vials	CSI Analytical Innovations	VT009M-1232	For a larger scale, use 50 mL falcons and evaporate the chloroform using rotary evaporator.
9mm Screw Cap	CSI Analytical Innovations	C395R-09LC	

C. Reagents required for step 3 (inner and feeding reaction mixtures):			
HEPES	Spectrum	H1089	1 M HEPES-KOH (pH = 8) - pH buffer
Potassium hydroxide (KOH)	Frutarom	55290	
1 M Magnesium acetate	Merck	1.05819.0250	Co-factor and negative charge stabilizor.
1 M Potassium acetate	Carlo Erba	470147	Negative charge stabilizor.
5.2 M Ammonium acetate	Merck	1.01116.1000	Stabilizes negative charge.
50% (w/v) Polyethylene glycol 6000 (PEG)	Merck	8.07491.1000	Increases the concentration of the macromolecules.
0.5 M 3-phosphoglycerate (3-PGA)	Sigma	P8877	Secondary energy source.
50 mM Amino acids mixture I	Sigma	LAA21-1KT	Amino acids additive. Contains: 50 mM of each of the following 17 natural amino acids - alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, and valine.
50 mM Amino acids mixture II	Sigma	LAA21-1KT	Amino acids additive. Contains: 50 mM of each of the following 3 natural amino acids - tryptophan, phenylalanine, and tyrosine.
100 mM Adenine triphosphate (ATP)	Sigma	A3377	Nucleotides & energy source.
50 mM Guanidine triphosphate (GTP)	Sigma	G8877	Nucleotides & energy source.
100 mM Uridine triphosphate (UTP)	ACROS ORGANICS	226310010	Nucleotides additive.
100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)	INALCO	INA-1758-1400	Genes expression induction.
2 M Sucrose	J.T. Baker	1933078	Generating a density gradient.
2 M Glucose	Sigma	16301	Generating a density gradient.
H_2O UltraPure Water (UPW)	Bio-Lab	2321777500	DNase & RNase free
S30-T7 lysate	_	_	Prepared at step 1. Source of transcription & translation components. Store at -80 °c, thaw on crashed ice just before usage.

Stock of DNA plasmid of choice	_	_	Contains the sequence for the requested protein. Under T7 promotor
D. Equipment required for step 4 (synthetic cells preparation)			
Floor incubator shaker or Thermomixer	MRC	TOU-120-2	Laboratory shaker incubator 450x450mm, 400rpm, 70 °C
	PHMT Grant Bio	PSC18	Thermomixer
Centrifuge	Thermo Scientific	75004270	(75003629) - TX-400 4 x 400mL Swinging Bucket Rotor. Suited for 15 mL sized tubes. Preferably swinging buckets. Should enable at least 1000 x g. Pre-cooled to 4 °C.
Table centrifuge	Thermo Scientific	75002420	(75003424) - 24 x 1.5/2.0mL rotor with ClickSeal. Suited for Eppendorf vials. Pre-cooled to 4 °C.
Vortex mixer	Scientific industries	SI-0256	
Crushed ice bucket	Bel-Art	M18848-4001	
Sterile 15 mL plastic tubes	Thermo Scientific	339651	
Sterilized 1.5 mL plastic tubes	Eppendorf	30120086	
Sterilized pipette tips	Corning		Sterilized by autoclave.

December 14, 2019

<u>To:</u> Nam Nguyen, Ph.D, Manager of Review, JoVE
<u>From:</u> Avi Schroeder, PhD, Department of Chemical Engineering
Technion – Israel Institute of Technology, Haifa 32000, Israel
avids@technion.ac.il

<u>Re:</u> "A Simple Protocol for Preparing Protein Producing Synthetic Cells and Cell-Free Bacterial Extracts using Liposomes and Emulsion Transfer"
Manuscript number: JoVE60829R1

Dear Dr. Nguyen,

Please allow us to thank you for the comments regarding our manuscript. We corrected the typos and performed additional editing of the text. All the materials and equipment (with the catalog numbers) are now described in Table of Materials. Following yours instructions we reduced the attached tables and the remaining describe compositions of the solutions used in the article.

Attached please find a 'track changes' copy of the revised manuscript.

We hope you find this version of the revised paper suitable for publication in this journal.

Warmly yours,

Avi Schroeder, PhD
Department of Chemical Engineering
Technion – Israel Institute of Technology, Haifa 32000, Israel

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