Title

High-Throughput Experimentation Using Cell-Free Protein Synthesis Systems

Authors

Meyer, Conary

Zhou, Chuqing

Fang, Zecong

et al.

Publication Date

2022

DOI

10.1007/978-1-0716-1998-8_7

Peer reviewed

Published in final edited form as:

Methods Mol Biol. 2022 ; 2433: 121–134. doi:10.1007/978-1-0716-1998-8_7.

High-Throughput Experimentation Using Cell-Free Protein Synthesis Systems

Conary Meyer[1], **Chuqing Zhou**[1], **Zecong Fang**[1,2,3], **Marjorie L Longo**[4], **Tingrui Pan**[1,2,3,5,6], **Cheemeng Tan**[1]

[1]Department of Biomedical Engineering, University of California, Davis, CA, USA.

[2]Institute of Biomedical and Health Engineering, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.

[3]Shenzhen Engineering Laboratory of Single-Molecule Detection and Instrument Development, Shenzhen, China.

[4]Department of Chemical Engineering, University of California, Davis, CA, USA.

[5]Suzhou Institute for Advanced Research, University of Science and Technology of China, Suzhou, China.

[6]Department of Precision Machinery and Precision Instrumentation, University of Science and Technology of China, Hefei, China.

Abstract

Cell-free protein synthesis can enable the combinatorial screening of many different components and concentrations. However, manual pipetting methods are unfit to handle many cell-free reactions. Here, we describe a microfluidic method that can generate hundreds of unique submicroliter scale reactions. The method is coupled with a high yield cell-free system that can be applied for broad protein screening assays.

Keywords

High throughput; Cell-free protein synthesis; Droplet printing; Automation; Protein screening; Microfluidic adaptive printing; Low-volume liquid handling

1 Introduction

Cell-free protein synthesis (CFPS) systems have gained tremendous interest from the development of applications including the synthesis of toxic and challenging proteins [1, 2], high-throughput screening [3], a priori prediction of genetic circuits [4, 5], in vitro metabolic pathways construction for biomanufacturing [6], and noncanonical amino acid incorporation [7]. While substantial progress has been made in this field, the application of these systems often requires high-throughput exploration of numerous parameters. These parameters include a wide range of different salt, reactant, and protein concentrations, leading to millions of possible combinations [8, 9]. Extensive exploration of this parameter space is expensive, error-prone, and prohibitively laborious using conventional pipetting methods [10].

To address the shortcomings of traditional liquid handling, numerous automated liquid handling robots have come to market (e.g., Fluent™ from Tecan and the OT-2™ from Opentrons). While these systems can minimize the involvement of researchers, they still incur the issue of inaccuracy at submicroliter volumes. This issue requires precious and expensive biological reagents to be used at a scale far greater than is necessary. Microfluidic adaptive printing technologies have been developed to allow for ultralow-volume liquid handling, effectively overcoming this critical problem [11-14]. These systems have been utilized in several examples of combinatorial screens since their inception [15-17]. Recently, a new pipette-free robotic-dispensing interface utilizing a simple microfluidics-enabled container has been developed [18]. The microfluidic cap-to-dispense (μCD) system integrates high accuracy robotic motion and droplet dispensing with nanoliter resolution to test hundreds to thousands of unique reactions rapidly and accurately.

The core of the μCD system is a disposable multilayer microfluidic container composed of a 3D printed reservoir linked to a microfluidic printing nozzle with two pneumatic control channels that can be reversibly connected to a robotic arm. This robotic arm is customized to enable reliable linking between the pneumatic controllers and the microfluidic device. The modular and disposable design of the μCD system allows for the inclusion of limitless reagents in a multicomponent experiment as the microfluidic devices can be swapped in and out without the risk of cross-contamination.

We will detail the setup and use of the μCD system, along with the production of the recently developed highly productive CFPS system described in [19]. This novel CFPS system utilizes a coculture of seven different strains, each overexpressing a subset of the eleven translation factors [20]. This overexpression both increases the concentration of the components needed for efficient translation and triggers a global proteome shift, which adapts the CFPS system to be more amenable to protein synthesis. The construction of the required strains and the method of whole-cell extract preparation are described below. However, this system can be utilized with any form of a cell-free system or in other applications requiring the manipulation of submicroliter volumes.

2 Materials

2.1 Strain and Plasmid Construction for Whole-Cell Extract

1. BL-7S plasmids (Addgene https://www.addgene.org/Cheemeng_Tan/).
2. pLysS plasmid (Novagen, cat# 69659).
3. BL21(DE3) chemically competent cells.

2.2 Whole-Cell Extract Preparation

1. 2×YTP: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride, 7 g/L Sodium phosphate dibasic, 3 g/L disodium phosphate monobasic.
2. 2×YTP agar plates: 2×YTP supplemented with 15 g/L agar.
3. Washing buffer: 10 mM Tris acetate, 14 mM Magnesium acetate, 60 mM Potassium Gluconate, pH to 7.6 using Sodium hydroxide.

4. BL21(DE3) pLysS Chemically Competent.
5. Sonication buffer: Washing buffer supplemented with 2 mM Dithiothreitol.
6. Carbenicillin (Carb).
7. Chloramphenicol (Cam).
8. Kanamycin (Kan).
9. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
10. Tabletop centrifuge.
11. 250-mL flat bottom centrifuge bottles.
12. Plate reader.
13. Q125 Sonicator with a 2-mm diameter probe (Qsonica model CL-18).

2.3 Preparation of Cell-Free Reaction Supplement

1. Amino acid solutions as indicated in Table 1, mixed together (*see* Notes 1 and 2).
2. Stock solutions specified in Table 2, mixed together to make the cell-free reaction supplement.

2.4 Cell-Free Reaction Supplement Preparation

1. Ribonucleotide triphosphates (ATP, GTP, UTP, and CTP).
2. Folinic acid.
3. *E. coli* tRNA mixture from *E. coli* MRE600.
4. 20 standard amino acids.
5. Nicotinamide adenine dinucleotide (NAD).
6. Coenzyme-A.
7. Spermidine.
8. Potassium glutamate.
9. Magnesium glutamate.
10. HEPES.
11. Creatine phosphate.
12. Creatine kinase.
13. Cyclic adenosine monophosphate (cAMP).
14. Polyethylene glycol 8000 (PEG8000).
15. Dithiothreitol.

2.5 Printing Platform Equipment and Consumables

1. PMMA sheets (75 μm thick).
2. Double-sided adhesive membrane (80 μm thick).
3. CO2 laser cutter.
4. 85 μm nozzle in 75 μm thick PMMA.
5. SLA 3D printer (Form 3 Formlabs).
6. Clear Resin (Formlabs).
7. Analytical Balance (U.S Solid 1 mg or ideally 0.1 mg precision).
8. Robotic arm (Dobot Magician).
9. Two mini solenoid valves (LHDA1221111H, Lee Co.)
10. Manifold (Lee Co.)
11. Automated pressure controller (PreciGenome Pressure/Flow controller light version, PG-MFC-LT2CH-X).
12. Microcontroller (Arduino Uno).
13. 12.5 V Power supply (BK precision programmable DC power supply)
14. Thin tubing (1/16″ and 1/8″ Tygon® S3™ E-3603 Non--DEHP Laboratory Tubing).
15. Circuit components.
    a. Diode 1 N4005.
    b. Zener Diode 1 N4757 51 V.
    c. Transistor MJH11022G.
    d. Resistor 330 Ω.
    e. Connector wires.
    f. Breadboard.

3 Methods

3.1 Construction of BL-7S Strains

1. Order the plasmids listed in Table 3 from Addgene.
2. Purify the plasmids listed using a standard miniprep plasmid purification kit.
3. Transform the plasmids along with pLysS into BL21(DE3) chemically competent cells. Thaw 50 μL of chemically competent cells on ice for 10 min. Add 1 ng of each plasmid to the cells and incubate on ice for 30 min. Heat shock at 42 °C for exactly 10 s and return to ice for 5 min. Recover the cells by adding 950 μL of room temperature SOC to the solution. Incubate for 60 min at 37 °C with

shaking. Mix the tubes and streak onto 2×YTP agar plates containing 100 μg/mL Carb, 34 μg/mL Cam, and 30 μg/mL Kan. Incubate the plates overnight at 37 °C.

3.2 Preparation of BL-7S Whole-Cell Extract

1. Inoculate 3 mL of 2×YTP supplemented with 100 μg/mL Carb, 34 μg/mL Cam, and 30 μg/mL Kan in a 15-mL culture tube with a single colony from each fresh plate.
2. Incubate the cultures overnight at 37 °C with shaking at 250 rpm.
3. Combine the cultures using the percentages specified in Table 3 and maintain a final OD600 of 1.0 and transfer 1.8 mL of the mixture to 2 L Erlenmeyer flask with 300 mL of 2×YTP supplemented with 100 μg/mL Carb and 30 μg/mL Kan (*see* Note 3).
4. Incubate the flask at 30 °C with 250 rpm shaking until the absorbance at 600 nm (OD600) of the culture reaches 0.15 (*see* Note 4). Once the desired concentration is reached, induce protein expression using a final concentration of 0.5 mM IPTG.
5. Continue to incubate the flask until the OD600 reaches 1.0 (*see* Note 4).
6. Transfer the cultures to 300 mL to flat bottom centrifugation bottles.
7. Centrifuge at 4000 × *g* for 20 min at 4 °C.
8. Decant the supernatant of each tube and resuspend with 20 mL of Wash Buffer (*see* Note 5).
9. Repeat the wash step.
10. Weigh a 50-mL conical tube for each 300 mL of culture and transfer the resuspended culture to the tubes.
11. Centrifuge at 4000 × *g* for 10 min at 4 °C.
12. Decant the supernatant (*see* Note 6).
13. Weigh the pellet and subtract the original mass of the tube to find the pellet mass.
14. Resuspend the pellet with 1 mL of Sonication Buffer per 1 g of wet cell mass (*see* Note 7).
15. Aliquot 500 μL of the resuspended pellet to 1.5-mL microfuge tubes.
16. Sonicate the cells at a frequency of 20 kHz and 50% amplitude while the tube is inside an ice-water slurry. Sonication is carried out with the Q125 Sonicator in 10 s ON/10 s Off intervals until the input energy is about 1000 J. This generally takes 27 cycles (*see* Note 8).
17. Centrifuge the cell lysate at 12,000 × *g* for 20 min at 4 °C.
18. Collect the supernatant and aliquot into individual tubes.
19. Incubate the tubes at 30 °C for 30 min (*see* Note 9).

20. Store the aliquots at −80 °C.

3.3 Printing Platform Assembly

The printing platform is comprised of several different components. The three-dimensional positioning and loading of printer heads are achieved using the Dobot Magician robotic arm with the gripper attachments. The printing and refuel pressures are regulated by the PreciGenome pressure controller. The Arduino microcontroller unit (MCU) and valves are used to apply the pressures in bursts for printing and refueling. The assembled system is shown in Fig. 1. The code, which is freely available at [https://github.com/ccmeyer/printing-platform], integrates and controls all these components for simple and robust use.

1. The required files for 3D printing and laser cutting the necessary components to construct the system are also available at [https://github.com/ccmeyer/printing-platform] (*see* Note 10).
2. Connect the gripper attachment to the Dobot and attach the 3D printed gripper adapters to the end effector.
3. Screw each valve into the manifold and attach it to the Dobot near the gripper (*see* Note 11).
4. Connect the gripper adapters to the manifolds using thin tubing. Then connect the manifold inlets to the corresponding pressure outlets on the PreciGenome pressure controller.
5. Construct the circuit shown in Fig. 2 and connect the Arduino, the two valves, and the power source to it (*see* Note 12).
6. Set the voltage of the power source to 12.0 V or the required voltage for the valves.
7. Laser-cut the provided template and place it around the Dobot to provide consistent placement of the well plate and balance in the Dobot's Cartesian coordinate system.
8. Connect the robotic arm, the pressure controller, and the Arduino to your computer.
9. Upload the provided Arduino program to the Arduino.
10. 3D-print the tube stand and place it on top of the scale.

3.4 Printer Head Fabrication

1. To fabricate the layer with the small nozzle, a UV-laser is used to ablate a small hole (65–85 μm diameter) into a PMMA sheet (*see* Notes 13 and 14).
2. The channel layer can be fabricated using a CO_2 laser to ablate microchannels in double-sided membrane adhesive. The chosen channel width was 200 μm (*see* Note 15).

3. The bulk component of the printer head, which contains the connections to the pressure inlets and the reagent reservoir, is fabricated by 3D printing (*see* Note 16). The Solidworks models are available on GitHub (*see* Note 17).

4. The full printer head is assembled stepwise. Peel off one side from the adhesive layer containing the channel and adhere it to the bottom of the 3D printed component. Line up the entry and exit holes of the channel with the corresponding openings on the 3D printed part.

5. Peel off the other side of the adhesive layer and place the nozzle layer on top. Ensure that the small nozzle is lined up with the exit hole of the channel. (*see* Note 18).

3.5 Printing Calibration

Due to the inherent variability between the physical properties of different reagents and imperfections in the fabrication process, the flow resistance through the channel and the nozzle will vary. If not accounted for, the droplet volume will differ from the intended volume. This section details how to rapidly calibrate the printer head with the desired reagent to achieve printing with nanoliter precision.

3.5.1 Calibration Theory

a. The fundamental idea of this calibration process is to leverage the fixed and tunable parameters to both determine and then account for the unknown parameters. The parameters of the system are Unknown—Channel and nozzle resistances; Fixed—Overflow chamber volume; Tunable—Refuel and printing pressures.

b. The unknown channel and nozzle resistances can be calculated using a simple procedure. Using the known dimensions of the overflow channel shown in Fig. 3, the volume displaced during the refuel and printing steps can be measured by monitoring the fluid level in the channel. The displaced volume and the known magnitude and duration of the applied pressure can be used to calculate the total flow of the system. The droplets that are ejected during the printing step are collected such that the volume dispensed can be calculated from the measured mass. This volume can be used to calculate the partial flow out of the nozzle. Using Eqs. 1-3 the individual resistances can be calculated using the magnitude of the pressure and the time the pressure was applied. In the equations below R represents resistance, P is pressure, t is time the pressure is applied, V is volume, ρ is density, and m is mass.

$$R_{\text{channel}} = \frac{P_{\text{refuel}}\, t_{\text{refuel}}}{V_{\text{chamber}}} \tag{1}$$

$$R_{\text{total}} = \frac{P_{\text{print}}\, t_{\text{pulse}}}{V_{\text{chamber}}} \tag{2}$$

$$R_{\text{nozzle}} = \frac{P_{\text{print}}\, t_{\text{pulse}} \rho_{\text{reagent}}}{m_{\text{droplet}}} \quad (3)$$

c. Once the resistances are known, the refuel and printing pressures can be adjusted to both allow for the generation of the desired droplet volume and ensure that the flow out of the overflow channel matches the flow in for continuous printing. The desired pressures can be calculated using Eqs. 4 and 5 where f_{print} represents the frequency of printing and t_{width} represents the duration of each printing pulse.

$$P_{\text{print}} = \frac{R_{\text{nozzle}} V_{\text{ejected}}}{f_{\text{print}} t_{\text{width}}} \quad (4)$$

$$P_{\text{refuel}} = \frac{P_{\text{print}} t_{\text{print}} R_{\text{channel}}}{R_{\text{total}}(1 - t_{\text{print}})} \quad (5)$$

3.5.2 Method for Individual Printer Head Calibration

1. Measure the density of the reagent by finding the mass of 100 μL of sample. Divide the number of milligrams by the volume dispensed to find the density in mg/μL.

2. Pipet the desired amount of reagent into an assembled printer head and load the printer head into the printing assembly (*see* Note 19).

3. Place the reagent tube into the tube holder on top of the scale and tare the scale. Position the Dobot holding the printer head directly above the tube and initiate the calibration protocol in the provided API.

4. Start by filling the printer head overflow chamber by applying refuel pressure until the liquid level reaches the first notch in the printer head. This is where the calibration starts.

5. Continue to apply refuel pressure until the overflow chamber fills to the second notch and hit continue.

6. Apply printing pulses until the liquid level in the overflow chamber reaches the first notch.

7. Repeat this process two more times to get replicates of the measurements.

8. Enter the new mass into the program, and it will automatically calculate and display the resultant resistances for the flow into and out of the overflow chamber as well as the nozzle resistance.

9. The program will then determine the needed refuel and printing pressures needed to continuously print the desired droplet volume with that printer head and reagent (*see* Note 20).

4 Notes

1. The different solutions used in the resuspension help to dissolve the amino acids.
2. It is recommended that a static gun, such as the Zerostat 3 from Sigma-Aldrich, be used to remove excess static from the tubes and scale while weighing the amino acids.
3. We exclude Cam as it is responsible for the maintenance of the pLysS plasmid, which is not needed at this step.
4. The 600 nm absorbance measurement was taken in a plate reader with a clear bottom 96-well plate with 200 μL of culture. The path length is roughly 0.5 cm compared to the standard 1cm pathlength used in OD600 measurements. Samples were diluted such that the absorbance reading was under 0.25 to maintain linearity.
5. The wash step is carried out on ice by swirling the Wash Buffer around to resuspend the pellet.
6. After decanting the supernatant, the centrifugation step can be repeated to remove excess liquid.
7. Use a laboratory spatula with a rounded end to mechanically resuspend the pellet.
8. The cell paste will turn more transparent and slightly pink if using wildtype BL21(DE3). However, if using the BL-7S strains, it will not significantly change color or turbidity.
9. This is a run-off reaction to allow time for the ribosomes to finish reading through the RNA that they are bound to. It is not necessarily required.
10. To download all the files and scripts required to build and operate the system, it is recommended to clone the Git repository.
11. It is critical to have a tight seal between the manifold and the valve to ensure no loss of pressure.
12. Provide enough extra tubing and wires to run them along the arm to not restrict its motion while in use.
13. A range of nozzle diameters can be used to give different sized droplets.
14. The nozzles can be fabricated by the following companies: Jestar Mold Tech Co., Ltd. (China) and Micron Laser Technology Inc. (USA).
15. A wide range of channel widths can be used. Choose the width based on the desired channel resistance.
16. We used stereolithography (SLA) printing with surgical guide photosensitive resin to generate the printer heads and the gripper adapters.

17. It is critical that these printer heads are watertight, transparent, and have smooth channels for proper use. These qualities are very difficult to achieve with standard fused deposition modeling (FDM) printers as there are many imperfections at the junctions between layers.

18. Firmly press the layers down to make sure that there are no air pockets.

19. The standard printer head design holds 200 μL of liquid.

20. Depending on the available lasers, the nozzle size might differ from the ones used in this instantiation of the system. The difference in nozzle size will impact the available range of droplet volumes that are possible. The pressure will correlate linearly with the droplet volume. However, the pressure must stay high enough to pinch off the droplet, which sets the floor for the possible droplet size.

Acknowledgments

This work was partially supported by the National Science Foundation under Grant No. DMR—1806366 and the NIEHS UC Davis Superfund Project 5P42ES004699-30. The research was also partially supported by the ARCS fellowship to C. M.

References

1. Lim HJ et al. (2016) Cell-free synthesis of functional phospholipase A1 from Serratia sp. Biotechnol Biofuels 9:1–7 [PubMed: 26734071]
2. Thoring L, Dondapati SK, Stech M, Wüsten-hagen DA, Kubick S (2017) High-yield production of ‘difficult-to-express’ proteins in a continuous exchange cell-free system based on CHO cell lysates. Sci Rep 7:1–15 [PubMed: 28127051]
3. Contreras-Llano LE, Tan C (2018) High-throughput screening of biomolecules using cell-free gene expression systems. Synth Biol 3:ysy01
4. Chappell J, Jensen K, Freemont PS (2013) Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology. Nucleic Acids Res 41: 3471–3481 [PubMed: 23371936]
5. Sun ZZ, Yeung E, Hayes CA, Noireaux V, Murray RM (2014) Linear DNA for rapid prototyping of synthetic biological circuits in an Escherichia coli based TX-TL cell-free system. ACS Synth Biol 3:387–397 [PubMed: 24303785]
6. Karim AS, Jewett MC (2016) A cell-free frame-work for rapid biosynthetic pathway prototyping and enzyme discovery. Metab Eng 36: 116–126 [PubMed: 26996382]
7. Martin RW et al. (2018) Cell-free protein synthesis from genomically recoded bacteria enables multisite incorporation of noncanonical amino acids. Nat Commun 9:1–9 [PubMed: 29317637]
8. Borkowski O et al. (2020) Large scale active-learning-guided exploration for in vitro protein production optimization. Nat Commun 11: 1–8 [PubMed: 31911652]
9. Dopp BJL, Tamiev DD, Reuel NF (2020) Cell-free supplement mixtures: elucidating the history and biochemical utility of additives used to support in vitro protein synthesis in E. coli extract. Biotechnol Adv 37(1):246–258
10. Art M, Dufey V, Gast U, Gligor I, Koch L, Kubasch R (2016) The tip of the iceberg: how pipette tips influence results. Appl Note 354:1–22
11. Ding Y, Huang E, Lam KS, Pan T (2013) Microfluidic impact printer with interchangeable cartridges for versatile non-contact multiplexed micropatterning. Lab Chip 13: 1902–1910 [PubMed: 23525299]
12. Villarreal F et al. (2017) Multi-dimensional studies of synthetic genetic promoters enabled by microfluidic impact printing. Lab Chip 17: 2198–2207 [PubMed: 28613297]

13. Fan J et al. (2018) Dotette: programmable, high-precision, plug-and-play droplet pipetting. Biomicrofluidics 12:034107 [PubMed: 29861810]
14. Mao Y et al. (2018) High-precision digital droplet pipetting enabled by a plug-and-play microfluidic pipetting chip. Lab Chip 18: 2720–2729 [PubMed: 30014071]
15. Li J et al. (2018) Microfluidic print-to-synthesis platform for efficient preparation and screening of combinatorial peptide microarrays. Anal Chem 90:5833–5840 [PubMed: 29633611]
16. Li J et al. (2018) A plug-and-play, drug-on-pillar platform for combination drug screening implemented by microfluidic adaptive printing. Anal Chem 90:13969–13977 [PubMed: 30358386]
17. Ding Y et al. (2015) Microfluidic-enabled print-to-screen platform for high-throughput screening of combinatorial chemotherapy. Anal Chem 87:10166–10171 [PubMed: 26334956]
18. Wang J et al. (2019) Microfluidic cap-to-dispense (μCD): a universal microfluidic–robotic interface for automated pipette-free high-precision liquid handling. Lab Chip 19: 3405–3415 [PubMed: 31501848]
19. Contreras-Llano LE, Meyer C et al. (2020) Holistic engineering of cell-free systems through proteome-reprogramming synthetic circuits. Nat Commun 11:3138 [PubMed: 32561745]
20. Villarreal F et al. (2017) Synthetic microbial consortia enable rapid assembly of pure translation machinery. Nat Chem Biol 14:29–35 [PubMed: 29131146]

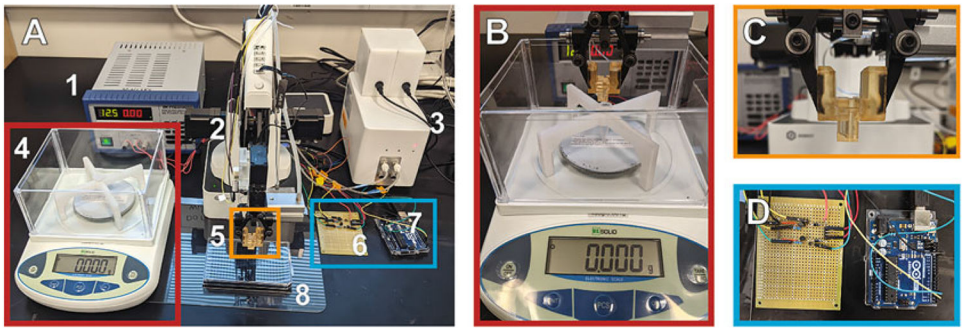

Fig. 1.

Diagram of the complete printing platform. (**a**) The image of the full platform. (1) 12.5 V DC power supply, (2) Dobot robotic arm, (3) Precigenome pressure regulator, (4) Analytical balance, (5) Gripper holding a printer head, (6) Valve control circuit, (7) Arduino Uno microcontroller, (8) Well plate. (**b**) Close-up of the printer head positioned over the reagent tube during calibration. (**c**) Dobot gripper with the 3D printed gripper adapters holding a printer head. (**d**) Valve control circuit connected to an Arduino

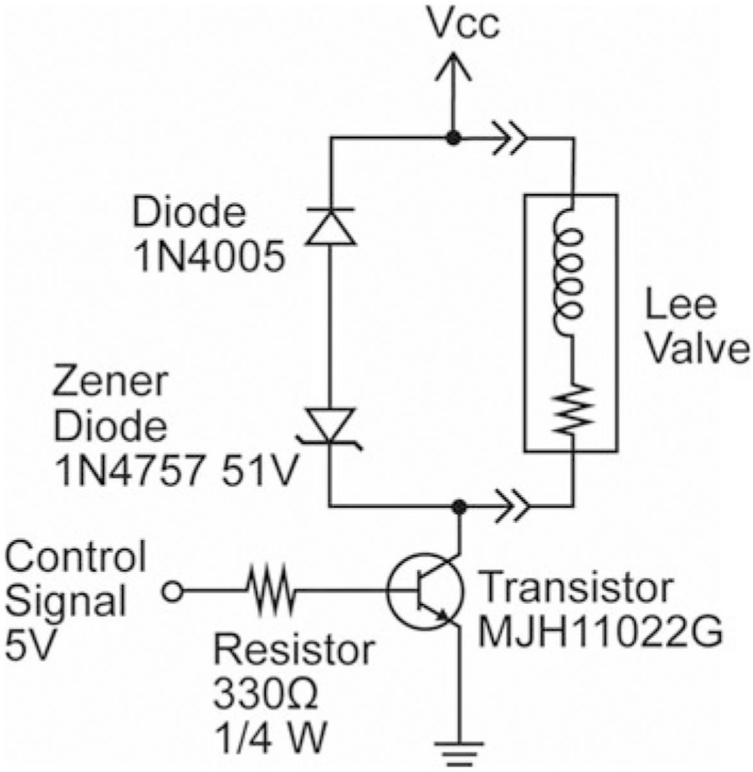

Fig. 2.

The Arduino circuit design. The circuit is used to regulate the valves that control the pressure flow

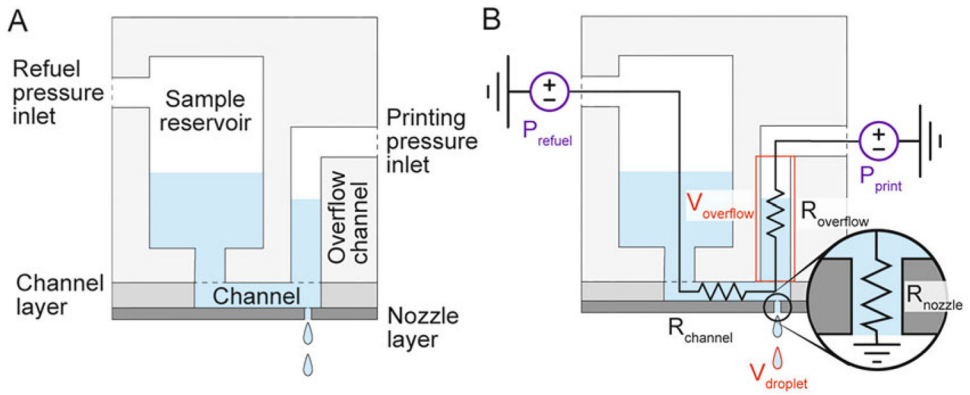

Fig. 3.

Schematic depicting the printer head design. (**a**) The components of the printer head. (**b**) Key control parameters of the system. Tunable parameters: purple. Fixed parameters: red. Unknown parameters: black

Table 1

Composition of Amino Acid mixture

Amino acid	Molecular weight	Stock conc. (M)	Solution	Volume (mL)	Mass (mg)
Alanine	89.09	0.01	Water	0.02	17.8
Arginine	210.66	0.01	Water	0.02	42.1
Asparagine	132.12	0.01	0.5 M HCl	0.02	26.4
Aspartic acid	133.1	0.01	0.5 M HCl	0.02	26.6
Cysteine	157.62	0.01	Water	0.02	31.5
Glutamic acid	147.13	0.01	1 M HCl	0.02	29.4
Glutamine	146.14	0.01	0.5 M HCl	0.02	29.2
Glycine	75.07	0.01	Water	0.02	15.0
Histidine	209.63	0.01	Water	0.02	41.9
Isoleucine	131.17	0.01	Water	0.02	26.2
Leucine	137.17	0.01	Water	0.02	27.4
Lysine	182.65	0.01	Water	0.02	36.5
Methionine	149.21	0.01	0.5 M HCl	0.02	29.8
Phenylalanine	165.19	0.01	5 M KOH	0.02	33.0
Proline	115.13	0.01	Water	0.02	23.0
Serine	105.09	0.01	Water	0.02	21.0
Threonine	119.12	0.01	Water	0.02	23.8
Tryptophan	204.23	0.01	0.5 M HCl	0.02	40.8
Tyrosine	181.19	0.01	1 MHCl	0.02	36.2
Valine	117.15	0.01	Water	0.02	23.4

Table 2

Composition of cell-free reaction supplement

Component	Molecular weight (g/mol)	Working concentration	Stock concentration
HEPES-KOH pH 7.6	238.3	0.05 M	2M
tRNA	N/A	0.17 mg/mL	17
Folinic acid	N/A	0.034 mg/mL	6.8 mg/mL
Magnesium glutamate	388.61	0.012 M	1.5 M
Potassium glutamate	203.23	0.18 M	5M
Polyethelene Glycol 8000	N/A	2%	40%
Dithiothreitol	154.25	0.002 M	1M
Spermidine	145.25	0.004M	0.5 M
Creatine phosphate	327.2	0.067 M	2 M
Creatine kinase	N/A	0.08 mg/mL	34.4 mg/mL
Nicotinamide adenine dinucleotide	663.4	0.33 mM	175 mM
Cyclic adenosine monophosphate	329.21	0.64 mM	640 mM
Coenzyme-A	767.53	0.26 mM	65 mM
20 amino acid mixture	N/A	2 mM	10 mM
ATP	507.18	1.2 mM	100 mM
GTP	523.18	1.2 mM	100 mM
CTP	483.16	0.8 mM	100 mM
UTP	484.14	0.8 mM	100 mM

Table 3

Strain construction and inoculation proportion

Strain number	pET15b backbone	pSC101 backbone	Relative density (%)
1	B1–4	B1–5	57.8
2	B7–5	B3–1	16.6
3	B7–1	B5–5	3.8
4	B4–1	B3–2	8.3
5	B4–4	B3–4	0.5
6	B1–3	B3–4	5.1
7	B7–1	Empty	7.9