



Published in final edited form as:

Methods Cell Biol. 2015 ; 128: 303–318. doi:10.1016/bs.mcb.2015.01.014.

## Engineering artificial cells by combining HeLa-based cell-free expression and ultra-thin double emulsion template

Kwun Yin Ho<sup>1,\*</sup>, Victoria L. Murray<sup>1,\*</sup>, and Allen P. Liu<sup>1,2,3,4</sup>

<sup>1</sup>Department of Mechanical Engineering, University of Michigan, Ann Arbor, Michigan, United States of America

<sup>2</sup>Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan, United States of America

<sup>3</sup>Cellular and Molecular Biology Program, University of Michigan, Ann Arbor, Michigan, United States of America

<sup>4</sup>Biophysics Program, University of Michigan, Ann Arbor, Michigan, United States of America

### Abstract

Generation of artificial cells provides the bridge needed to cover the gap between studying the complexity of biological processes in whole cells and studying these same processes in an *in vitro* reconstituted system. Artificial cells are defined as the encapsulation of biologically active material in a biological or synthetic membrane. Here, we describe a robust and general method to produce artificial cells for the purpose of mimicking one or more behaviors of a cell. A microfluidic double emulsion system is used to encapsulate a mammalian cell free expression system that is able to express membrane proteins into the bilayer or soluble proteins inside the vesicles. The development of a robust platform that allows the assembly of artificial cells is valuable in understanding subcellular functions and emergent behaviors in a more cell-like environment as well as for creating novel signaling pathways to achieve specific cellular behaviors.

### Keywords

Artificial cells; cell-free expression; double emulsion; lipid bilayer vesicle; synthetic biology; capillary microfluidics

### Introduction

Biological processes can be studied in the context of a whole cell or in biochemical reconstitution *in vitro*. Both methodologies have advantages and disadvantages. By using whole cells, the biological process can be studied and understood in a physiologically relevant environment. Often, this involves genetic manipulation or drug perturbation to the cells and monitoring cellular responses. Even without manipulation, the inherent complexity

\*These authors contributed equally to this work

of intracellular subsystems and signaling pathways gives rise to possible unknown factors influencing the components under study as proteins rarely play a single role in a single process. In order to simplify the study of intracellular events, researchers have been able to reconstitute many major subcellular functions *in vitro*, and this has led to deeper understanding of many processes. However, *in vitro* reconstitution of spatially organized processes has been more challenging as many of these processes are membrane-based and require an enclosed environment. Lipid bilayer vesicles provide an enclosed, defined environment and a robust platform to study desired biological functions. We believe an artificial cell platform provides a much-needed bridge between studying cell biology at the cellular level and *in vitro* reconstitution of subcellular functions. Here, our working definition of an artificial cell requires the encapsulation of biologically active materials by a membrane (biological or synthetic).

*In vitro* reconstitution allows precise control over the concentration of different proteins and cofactors and the results have provided implications for our understanding of numerous cellular processes, most notably of actin-based motility in cells. Previously, actin-based motility has been reconstituted using purified proteins (Loisel, Boujema, Pantaloni & Carlier, 1999). As the natural load for actin-driven processes is biological membrane, researchers began to incorporate supported lipid bilayers and giant unilamellar vesicles in their reconstituted systems (Co, Wong, Gierke, Chang & Taunton, 2007; Liu & Fletcher, 2006; Liu, Richmond, Maibaum, Pronk, Geissler & Fletcher, 2008). Taking the first steps toward building true artificial cells, purified proteins and various cofactors were encapsulated into liposomes and used to investigate actin network formation and bacterial cell division (Jiménez, Martos, Vicente & Rivas, 2011; Merkle, Kahya & Schwille, 2008; Osawa, Anderson & Erickson, 2008; Tsai, Stuhmann & Koenderink, 2011). With the advances made in cell free protein expression over the years (Carlson, Gan, Hodgman & Jewett, 2012), several groups have been able to encapsulate a bacterial cell free expression system inside liposomes and demonstrated expression of proteins within the liposome (Tawfik & Griffiths, 1998; Yu, Sato, Wakabayashi, Nakaishi, Ko-Mitamura, Shima et al., 2001). In 2004, Libchaber and co-worker made a further advance that would allow for expression of proteins over long periods of time by using reverse emulsion template to encapsulate cell free system that form vesicles in a feeding solution (Noireaux & Libchaber, 2004). With the extended expression time, genetic circuits with controlled expression of various genes could be monitored (Vincent, Roy, Jeremy, Hanna & Albert, 2005).

Generation of vesicles with high encapsulation capacity is an important step of building artificial cells. The encapsulation of a cell free expression system inside vesicles has generally been accomplished using the droplet emulsion transfer method (Noireaux & Libchaber, 2004), which was first developed by Pautot *et al.* (Pautot, Frisken & Weitz, 2003). The method can generate monodispersed and unilamellar lipid vesicles with the help of microfluidics (Hamada, Miura, Komatsu, Kishimoto, Vestergaard & Takagi, 2008; Hu, Li & Malmstadt, 2011; Nishimura, Suzuki, Toyota & Yomo, 2012), but the droplets usually break when crossing the oil-aqueous interface, resulting in a low yield. In addition, oil residue may remain in the vesicles during the emulsion transfer process and alter the incorporation of membrane proteins into the vesicles. More recently, with the help of

microfluidic techniques, a layer-by-layer assembly method was developed, which offered good control of the lipid composition in the two lipid leaflets and provided the feasibility to generate asymmetric lipid bilayer vesicles (Matosevic & Paegel, 2013). However, this method has a very low yield. The droplets often burst when a new layer is deposited onto the first layer. In 2008, the Weitz group developed another liposome generation technique called double emulsion template (Shum, Lee, Yoon, Kodger & Weitz, 2008). In this approach, lipid-stabilized double emulsions with a volatile middle oil phase were first generated, and vesicles then formed upon the evaporation of the oil. The microcapillary device could generate monodispersed double emulsions so that vesicles generated using double emulsion template are also monodispersed (A. S. Utada, Lorenceau, Link, Kaplan, Stone & Weitz, 2005). The time required for vesicles to form from double emulsions depends on the thickness of the middle oil phase in the double emulsions. As the technique in generating ultra-thin double emulsions has become more mature recently (Kim, Kim, Cho & Weitz, 2011), ultra-thin double emulsion template emerges as an attractive technique for generating monodispersed unilamellar vesicles with high yield (Arriaga, Datta, Kim, Amstad, Kodger, Monroy et al., 2014).

Our approach in building artificial cells comprises of the generation of monodispersed unilamellar vesicles by ultra-thin double emulsion template and protein expression by a HeLa-based cell-free expression system. HeLa-based cell-free expression components, including HeLa lysate, truncated GADD34, and T7 RNA polymerase, are encapsulated with plasmid DNA inside unilamellar vesicles by ultra-thin double emulsion template (Fig. 10.1). In this Chapter, we demonstrate the expression of a membrane protein, mechanosensitive channel of large conductance (MscL), and its insertion to the lipid membrane. The plasmid DNA was transcribed into messenger RNA (mRNA) and the mRNA was translated to form membrane protein in a single reaction mix. In this approach, the ultra-thin double emulsion template enables the monodispersed generation of artificial cells at high throughput. The HeLa-based cell-free expression system provides a versatile platform to express mammalian proteins, which may require post-translational modification. Our approach enables us to engineer functional artificial cells. The artificial cells can either perform cell-like functions or be used to reconstitute biological processes in a well-controlled and confined environment.

### 10.1 Generation of HeLa-based Cell-Free Expression System

Although *E. coli* cell free expression systems are the most widely used and commercially available, a mammalian system has been chosen as human proteins requiring post-translation modifications will be utilized in down-stream applications of these artificial cells and for more versatile applications. The cell free expression system described below was originally reported in 2006 by the Imataka lab and has been improved upon over the years (Machida, Mikami, Masutani, Mishima, Kobayashi & Imataka, 2014; Mikami, Kobayashi & Imataka, 2010; Mikami, Kobayashi, Masutani, Yokoyama & Imataka, 2008; Mikami, Kobayashi, Yokoyama & Imataka, 2006; Mikami, Masutani, Sonenberg, Yokoyama & Imataka, 2006).

### 10.1.1 Materials

GADD34 1-240 (stock concentration of 2.3  $\mu$ M)

T7 RNA Polymerase (stock concentration of 5  $\mu$ M)

HeLa lysate (Thermo Fisher Scientific)

Mix 1 [27.6 mM Mg(OAc)<sub>2</sub>, 168 mM K-HEPES (pH 7.5)]

Mix 2 [12.5 mM ATP, 8.36 mM GTP, 8.36 mM CTP, 8.36 mM UTP, 200 mM creatine phosphate, 7.8 mM K-HEPES (pH 7.5), 0.6 mg/ml creatine kinase, 0.3 mM amino acid mixture, 5 mM spermidine, 44.4 mM DTT]

pT7CFE1-CHis vector (Thermo Fisher Scientific)

MscL-GFP-pT7CFE DNA (stock concentration of 500 ng/ $\mu$ l)

GFP-pT7CFE DNA (stock concentration of 500 ng/ $\mu$ l) (Thermo Fisher Scientific)

Small unilamellar vesicles (SUVs): 18:1 (9-Cis) PC (DOPC) 1,2-dioleoyl-sn-glycero-3-phosphocholine, 18:1 PS (DOPS) 1,2-dioleoyl-sn-glycero-3-phospho-L-serine, 18:1 Liss Rhod PE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), cholesterol

Rehydration buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl]

100 nm membrane (Whatman Nuclepore Track-Etched)

Silica microspheres 5  $\mu$ m (Bangs Laboratories)

High salt buffer [10 mM Tris-HCl (pH 7.4), 250 mM NaCl]

### 10.1.2 Equipment

Microcentrifuge tubes

Glass test tubes

Desiccator

Plate Reader

Heat block

Extruder kit (Avanti Polar Lipids)

### 10.1.3 Methods

#### 10.1.3.1 Cell Free Expression and Protein Yield Determination

1. HeLa lysate was obtained from a commercial source (Thermo Fisher Scientific). Lysate can also be prepared in lab (Mikami, Kobayashi & Imataka, 2010).
2. Both GADD34 1-240 (GST and HIS tagged) and T7 RNA Polymerase (GST-tagged) DNA constructs were obtained from H. Imataka (University of Hyogo, Japan) and purified following previously established protocols (Mikami, Kobayashi, Machida, Masutani, Yokoyama & Imataka, 2010; Mikami et al., 2008).

GADD34 1-240 is needed to enhance dephosphorylation of eIF2 $\alpha$ , promoting protein synthesis. Briefly, an LB culture containing GADD34 1-240 (BL21DE3) was grown at 30°C until OD<sub>600</sub> reached 0.4 to 0.6 and IPTG was added to a final concentration of 0.1 mM to induce expression. Culture was grown overnight at 20°C and cells were harvested next morning. Clarified lysate was first run through a Ni-NTA column, washed and eluted with 250 mM imidazole buffer. Eluant was then run through a glutathione agarose column and protein was cleaved with PreScission Protease (GE). Protein was aliquoted and stored at -80°C. GST-tagged T7 RNA polymerase expression and purification started with a 1:100 dilution of overnight culture into fresh LB culture and allowing it to grow at 37°C until OD<sub>600</sub> reached 0.6 to 1.0. To induce expression of protein, IPTG was added to a final concentration of 0.1 mM to the culture and grown overnight at 25°C. Cells were harvested the next morning. Clarified lysate was run through a glutathione agarose column and protein was cleaved with PreScission Protease. Eluant was dialyzed overnight, aliquoted the next day and stored at -80°C.

3. Mix 9  $\mu$ l lysate, 2.25  $\mu$ l Mix 1 and 2.7  $\mu$ l GADD34 (to give a final concentration of 310 nM) in a microcentrifuge tube and incubate at 32°C for 10 minutes.
4. Add 2.25  $\mu$ l Mix 2, 1  $\mu$ l DNA (500 ng) and 1.8  $\mu$ l T7 RNA polymerase (to give a final concentration of 450 nM) and incubate at 32°C for 3 hours.
5. To ascertain protein production, add reaction mixture (with and without GFP DNA) to two wells of 384-well plate. Using a plate reader, monitor GFP expression by exciting wells at 482 nm and collecting emission at 512 nm every 5 minutes for 3 hours.
6. Using serially diluted purified recombinant GFP, collect emission data of samples to produce standard curve (data not shown) and determine how much GFP was produced in the expression system (see Fig. 10.2A).

#### 10.1.3.2 Small Unilamellar Vesicle (SUV) Generation

1. Mix lipids together in a glass test tube with a desired mole percentage in chloroform (e.g. DOPC:cholesterol:DOPS:DOPE-lissamine = 60:30:9.5:0.5) and final lipid mass (excluding cholesterol) equals 12.5 mg. Other lipid compositions can be used depending on the needs of the experiment.
2. Add the lipid solution to a glass test tube and place under a stream of argon gas to evaporate chloroform, forming a lipid film. (Note: Inert gas avoids the oxidation of phospholipids.)
3. Place the glass tube in a desiccator under vacuum for 30 minutes.
4. Add 250  $\mu$ l rehydration buffer (50 mg/ml final lipid concentration), vortex for 1 minute and then incubate at 60°C for ten minutes. Repeat the vortex and incubation steps twice more.
5. Using the Avanti mini-extruder with a 100 nm membrane, push the lipid mixture through the membrane 11 times.

6. Store the SUVs at 4°C for up to two weeks.

### 10.1.3.3 Validation of membrane protein insertion in liposome (Confocal imaging)

1. Prepare lipid coated beads: Mix 20  $\mu$ l SUV stock with 20  $\mu$ l silica microspheres and 20  $\mu$ l high salt buffer in a microcentrifuge tube.
2. Vortex for 10 seconds. Wash 3X with 1 ml DI-water by adding 1 ml water to the tube and then vortexing for 10 seconds. Aspirate water and be careful with the bead pellet.
3. Resuspend bead pellet in 40  $\mu$ l DI-water.
4. When preparing cell free expression mixture in subsection 10.1.3.1, follow step 3 and then in step 4 add soluble GFP-pT7CFE in one tube and MscL-GFP-pT7CFE DNA to another tube, then add 2  $\mu$ l lipid coated beads to each tube; mix gently.
5. After the 3 hours incubation at 32°C, gently wash lipid coated beads 3X with 1 ml PBS. Load into chamber slide (coverslip adhered to slide using two strips of double sided tape, sealed with VLAP on remaining two sides after loading sample).
6. Image slide using 560 nm to excite rhodamine lipids (emission ~583 nm) and 488 nm to excite GFP (emission ~507 nm). In Fig. 10.2B, lipid coated beads expressed with soluble GFP did not show any incorporation of protein into the lipid bilayer. However, lipid coated beads expressed with GFP-tagged MscL showed insertion of protein into the bilayer.

## 10.2 Building Ultra-thin Double Emulsion Device

Double emulsions can be generated in a glass microcapillary device, which was initially developed by Utada et al. (A. S. Utada et al., 2005). The microcapillary device was modified so that double emulsions with ultra-thin shell can be generated (Kim et al., 2011). The device is built based on the assembly of glass capillaries on a glass slide. The flow of three different phases inside the device can be accurately controlled independently by three syringe pumps. When the flow is adjusted correctly, the fluid will break and form aqueous-in-oil-in-aqueous double emulsions in the dripping regime (A. S. Utada, Chu, Fernandez-Nieves, Link, Holtze & Weitz, 2007; Andrew S. Utada, Fernandez-Nieves, Stone & Weitz, 2007).

### 10.2.1 Materials

- Glass slides [75  $\times$  25 mm]
- Standard cutter with carbide wheel (Glass cutter)
- 5 minute epoxy (Devcon)
- Syringe needles [20G]
- Square capillaries [ID: 1.05 mm  $-0/+$  0.1 mm]
- Round capillaries [OD: 1 mm]

Trichloro(1H,1H,2H,2H-perfluorooctyl)silane 97% (Sigma) (referred to as silane onwards)

### 10.2.2 Equipment

Pipette puller (Sutter Instrument Co. P-87)

Sand paper (Fandeli B-99)

Optical microscope

Bunsen burner

### 10.2.3 Methods

1. Build a device platform using several glass slides. Glass slides are glued together using 5 minute epoxy (Fig. 10.3A(i)).
2. Customize the length of a square capillary and glue it on the platform using 5 minute epoxy (Fig. 10.3A(ii)).
3. Use a pipette puller to pull a round capillary into a tapered shape.
4. Modify the inner diameter of two tapered capillaries using sand paper (Fig. 10.3A(iii)). Make sure two tapered capillaries have different inner diameters for better generation of double emulsions. The inner diameters of the injection tube and collection tube are 50  $\mu\text{m}$  and 80  $\mu\text{m}$  respectively.
5. Treat the inner glass surface of the smaller tapered capillary with silane for at least one hour.
6. Remove excess silane and dry the inner glass surface with compressed air after silane treatment. Make sure no silane is left at the capillary tip.
7. Insert two round capillaries into the square capillary from each side (Fig. 10.3A(iv)).
8. Align the tips of two round capillaries under an optical microscope at 10x magnification and glue the capillaries to the platform using 5 minute epoxy (Fig. 10.3A(v)).
9. Pull a round capillary above a fire flame to form two long and thin pipettes (Fig. 10.3A(vi)). The outer diameter of the pulled pipette should be around 200  $\mu\text{m}$ .
10. Insert one pulled pipette into the back of the injection tube.
11. Align the tip of the pulled pipette to the center of the injection tube with an optical microscope (Fig. 10.3A(vii)).
12. Cut the syringe needles to allow openings and glue them on the platform using 5 minute epoxy at the end of each round capillary and square capillary (Fig. 10.3A(viii)).
13. Check the device and make sure there is no leakage. Use 5 minute epoxy to seal any leakage locations.

### 10.3 Encapsulation of HeLa-based Cell-Free Expression System in Lipid Bilayer Vesicles

To form vesicles, the ultra-thin double emulsions formed consist of a volatile oil as the middle phase separating the inner encapsulated aqueous phase and the outer aqueous phase. Phospholipids dissolved in the middle oil phase tend to assemble at the aqueous-oil interface and stabilize the emulsions. When oil begins to evaporate, it will de-wet the lipid interface. A vesicle will form when all the oil evaporates.

Artificial cells are engineered by encapsulating HeLa-based cell-free expression system with plasmid encoding the protein of interest. The cell-free expression components were mixed with the plasmid DNA on ice before being encapsulated in the ultra-thin double emulsions as the inner phase using the glass microcapillary device described in section 10.2. Expression of MscL and GFP are demonstrated using our approach.

#### 10.3.1 Materials

Poly(vinyl alcohol) (PVA)

UltraPure DNase/RNase free distilled water (Life Technologies)

HeLa-based cell-free expression components

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)

Cholesterol (Chol)

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE)

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2000)

Chloroform

Hexane

Brij-35

Potassium chloride

HEPES

Glycerol

Triton X-100

Glucose

#### 10.3.2 Equipment

Ultra-thin double emulsion device

3 syringe pumps

3 syringes (250  $\mu$ l, 2.5 ml, 10 ml)

Syringe needles

Optical microscope

High speed camera

Polyethylene micro tubing [ID: 0.034" × OD: 0.052"]

Glass test tube

### 10.3.3 Methods

1. Dissolve PVA into UltraPure water to a concentration of 15% (w/v). Stir the solution at 70°C overnight. Filter (0.22 µm) the solution before use.
2. Mix lipids together in a glass test tube with a desired mole percentage in chloroform (e.g. DOPC:Chol:Rho-PE:PEG2000 = 65.8:30:0.2:4) for a total 7.2 mg of lipids.
3. Dry the lipids by evaporating chloroform using an inert gas (e.g. Argon) until all chloroform evaporates.
4. Further evaporate the chloroform in a desiccator under vacuum for one hour.
5. During the evaporation of chloroform, prepare the solutions for inner phase and outer phase. Inner phase consists of HeLa lysate and the various accessory components (mix according to subsection 10.1.3.1) with 2% (w/v) PVA and 0.04% Brij-35. Outer phase contains 10% (w/v) PVA, 30 mM HEPES-K (pH = 7.7), 85 mM KCl, 2% glycerol, 0.015% Triton X-100 and 30 mM glucose.
6. After the lipids are completely dried, add 1.2 ml of chloroform and hexane to the glass test tube to form the middle phase in a volume ratio of 36:64 to a final lipid concentration of 6 mg/ml.
7. Fill the ultra-thin double emulsion device with UltraPure water to remove all the air bubbles inside.
8. Transfer the three phases to three syringes and connect them to microtubings through syringe needles and to the syringe pumps (Fig. 10.3C).
9. Start the pump and flow the three phases into the device at flow rates of 3000 µl/hr.
10. When the three phases have entered the device, adjust the flow rates of both inner and middle phases to 300 µl/hr.
11. If the surface treatment and alignment are both good, ultra-thin double emulsions with cell free expression components encapsulated will form.
12. Collect the solution at the outlet.
13. Warm up the collected solution to 32°C for both protein expression and solvent evaporation.

## 10.4 Results and Discussion

The encapsulation of HeLa-based cell-free expression system in lipid bilayer vesicles using ultra-thin double emulsion template was used to generate vesicles expressing cytosolic GFP

and mechanosensitive membrane proteins (MscL) with a C-terminal GFP fusion. The green fluorescent signal from the cytosolic GFP in the confocal images confirmed that the cytosolic GFPs were expressed and distributed uniformly inside the liposome (Fig. 10.4 (top)). In the experiment expressing MscL-GFP, fluorescent signal was detected at the membrane and demonstrated MscL insertion into the lipid bilayer vesicle (Fig. 10.4 (bottom)).

Our approach, in general, provides a general methodology for constructing artificial cells by incorporating cytosolic proteins or membrane proteins into the vesicles. Low concentrations of detergents are required to keep membrane proteins soluble inside the liposomes so that the membrane proteins can insert into the membrane. Since the artificial cell is expressing our proteins of interest, it can provide us with a platform to investigate biological processes in a simplified and well-controlled environment. The HeLa-based cell-free expression system in this approach provides the feasibility to reconstitute both mammalian and bacterial proteins in the artificial cell, so that the choice of proteins in the artificial cell is not limited by the expression system. However, there are inherent limitations of this enclosed expression system. First, reaction substrates will be limited and protein expression will only be sustainable for a few hours. Second, unless genetic circuits are employed, there will be no control over how much protein is expressed over time. Fortunately, based on the concentration of GFP produced in Fig. 10.1, we made ~71,000 copies of GFP in a vesicle with a 10  $\mu\text{m}$  diameter. This level of protein production nearly matches the median of protein copies per cell found in a study that quantified protein production on a genome-wide scale (Schwanhausser, Busse, Li, Dittmar, Schuchhardt, Wolf et al., 2011). A simple way to reduce protein production, if necessary, would be to add less DNA to reaction mix. The expression of protein is coupled with the encapsulation of the HeLa-based cell-free components inside the ultra-thin double emulsions. The evaporation of middle oil phase cannot be too fast because the double emulsions could burst and this would reduce the yield, but it has to be fast enough so that lipid membrane becomes available for the insertion of membrane proteins. Another critical part of the double emulsion template is the fabrication of double emulsion device. The tapered capillaries have to be coaxially aligned inside the square capillary, and the inner surface of the injection tube has to be treated such that it is sufficiently hydrophobic to prevent wetting of the capillaries by the aqueous phase and disturbing the fluid flow. A well fabricated double emulsion device can be used to generate ultra-thin double emulsions for over two months. The lipid vesicles generated through the ultra-thin double emulsion template are monodispersed and unilamellar. The combination of this method with the HeLa-based cell-free expression system will allow us to engineer artificial cells with various functions. However, the complete removal of organic solvent in the artificial cells could be a challenge of this approach depending on the exact application.

Plasma membrane proteins allow cells to sense the extracellular environment and initiate intracellular responses. Our model membrane protein, MscL, is found in bacteria and it opens when the membrane is under high tension. By reconstituting MscL into lipid bilayer vesicles, one can envision building an artificial cell that senses osmotic pressure and allow the influx of extracellular molecules that can activate downstream signaling pathways. By coupling multiple membrane proteins and cytosolic proteins inside a vesicle, we can engineer an artificial cell that is functional and can perform certain cellular functions. The

ultra-thin double emulsion template in this approach allows the generation of artificial cells in high throughput, which will be important for potential downstream applications of the artificial cells when they are used in functional tests *in vivo*. The combination of HeLa-based cell-free expression system and ultra-thin double emulsion template described in this Chapter enables the future engineering of functional artificial cells.

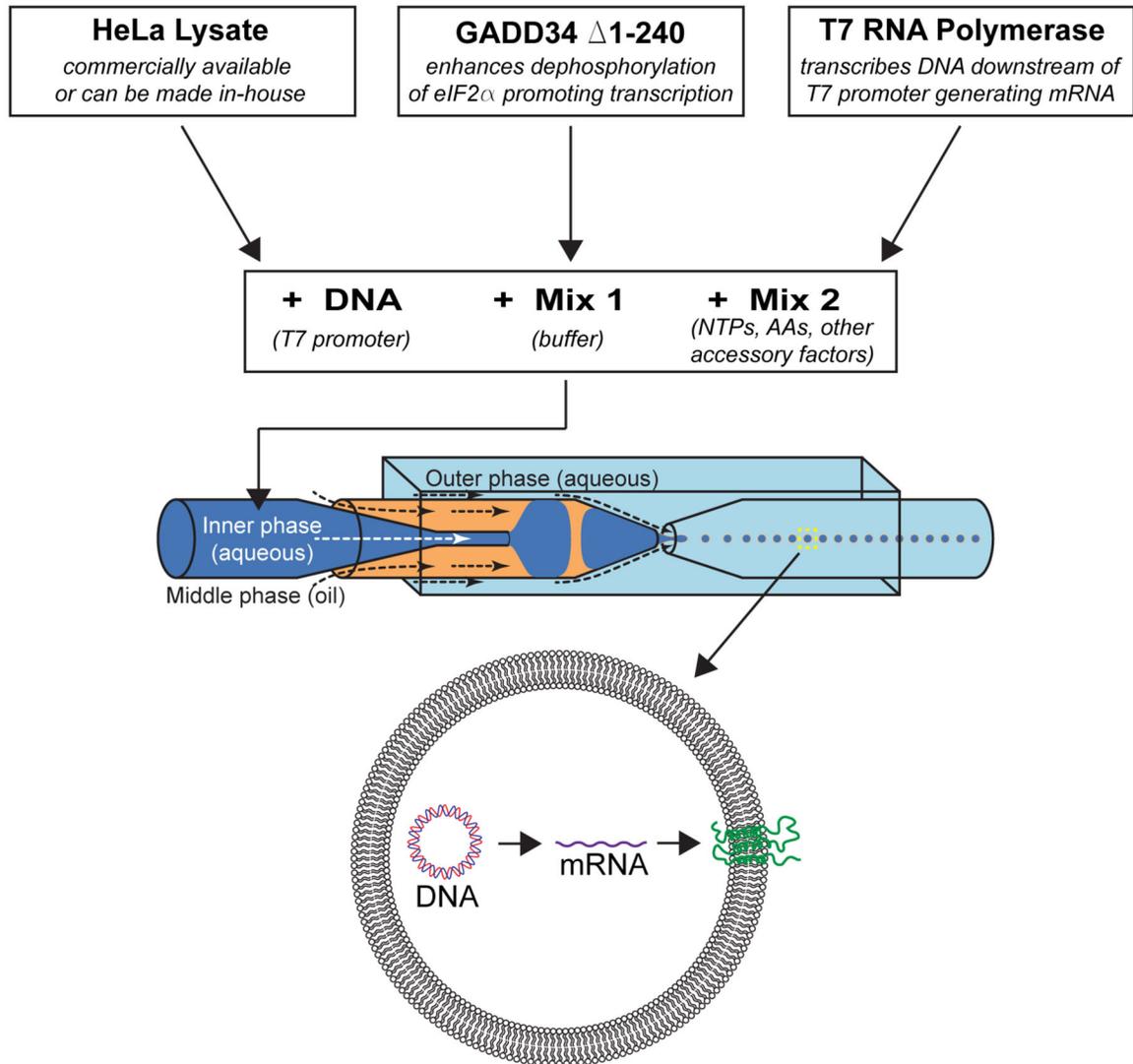
## Acknowledgments

We acknowledge Hirokai Imataka (University of Hyogo, Japan) for providing expression constructs for T7 RNA polymerase and GADD34 1-240 and assistance trouble shooting our cell free expression system. We thank all members of the Liu lab for helpful discussion. The work is supported by the NIH Director's New Innovator Award (DP2 HL117748-01).

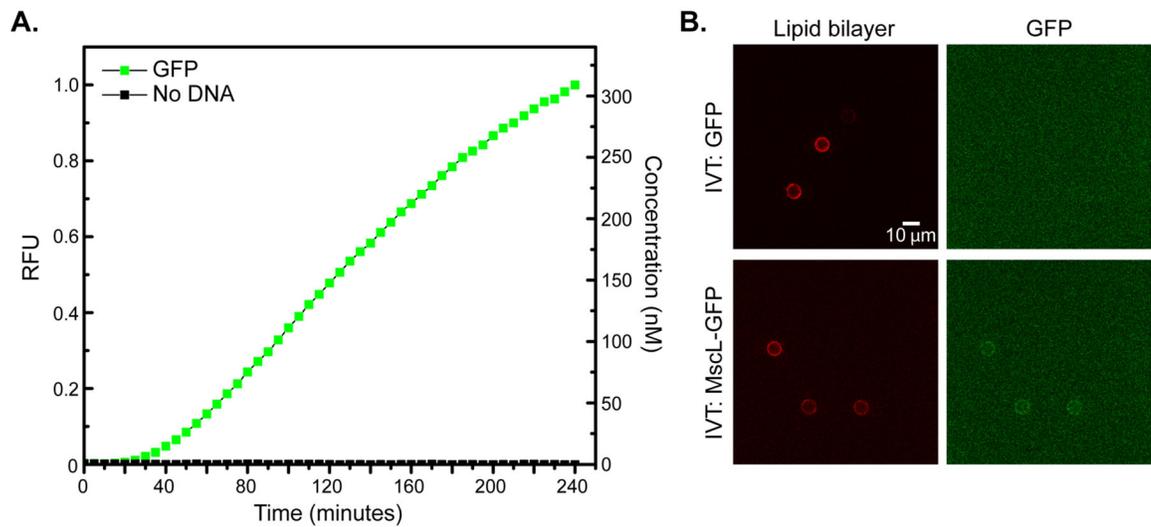
## References

- Arriaga LR, Datta SS, Kim SH, Amstad E, Kodger TE, Monroy F, Weitz DA. Ultrathin Shell Double Emulsion Templated Giant Unilamellar Lipid Vesicles with Controlled Microdomain Formation. *Small*. 2014; 10(5):950–956.10.1002/sml.201301904 [PubMed: 24150883]
- Carlson ED, Gan R, Hodgman CE, Jewett MC. Cell-free protein synthesis: Applications come of age. *Biotechnology Advances*. 2012; 30(5):1185–1194. <http://dx.doi.org/10.1016/j.biotechadv.2011.09.016>. [PubMed: 22008973]
- Co C, Wong DT, Gierke S, Chang V, Taunton J. Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. [Research Support, N.I.H., Extramural]. *Cell*. 2007; 128(5):901–913.10.1016/j.cell.2006.12.049 [PubMed: 17350575]
- Hamada T, Miura Y, Komatsu Y, Kishimoto Y, Vestergaard Md, Takagi M. Construction of asymmetric cell-sized lipid vesicles from lipid-coated water-in-oil microdroplets. *The journal of physical chemistry B*. 2008; 112(47):14678–14681.10.1021/jp807784j [PubMed: 18983183]
- Hu PC, Li S, Malmstadt N. Microfluidic fabrication of asymmetric giant lipid vesicles. *ACS applied materials & interfaces*. 2011; 3(5):1434–1440.10.1021/am101191d [PubMed: 21449588]
- Jiménez M, Martos A, Vicente M, Rivas G. Reconstitution and Organization of Escherichia coli Protoring Elements (FtsZ and FtsA) inside Giant Unilamellar Vesicles Obtained from Bacterial Inner Membranes. *Journal of Biological Chemistry*. 2011; 286(13):11236–11241.10.1074/jbc.M110.194365 [PubMed: 21257762]
- Kim SH, Kim JW, Cho JC, Weitz DA. Double-emulsion drops with ultra-thin shells for capsule templates. [10.1039/C1LC20434C]. *Lab on a Chip*. 2011; 11(18):3162–3166.10.1039/c1lc20434c [PubMed: 21811710]
- Liu AP, Fletcher DA. Actin Polymerization Serves as a Membrane Domain Switch in Model Lipid Bilayers. *Biophysical Journal*. 2006; 91(11):4064–4070. <http://dx.doi.org/10.1529/biophysj.106.090852>. [PubMed: 16963509]
- Liu AP, Richmond DL, Maibaum L, Pronk S, Geissler PL, Fletcher DA. Membrane-induced bundling of actin filaments. [10.1038/nphys1071]. *Nat Phys*. 2008; 4(10):789–793. [http://www.nature.com/nphys/journal/v4/n10/supinfo/nphys1071\\_S1.html](http://www.nature.com/nphys/journal/v4/n10/supinfo/nphys1071_S1.html). [PubMed: 19746192]
- Loisel TP, Boujema R, Pantaloni D, Carlier MF. Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. [Research Support, Non-U.S. Gov't]. *Nature*. 1999; 401(6753):613–616.10.1038/44183 [PubMed: 10524632]
- Machida K, Mikami S, Masutani M, Mishima K, Kobayashi T, Imataka H. A Translation System Reconstituted with Human Factors Proves that Processing Encephalomyocarditisvirus Proteins 2A and 2B Occurs in the Elongation Phase of Translation without Eukaryotic Release Factors. *J Biol Chem*. 2014; 289(10):6743–6750.10.1074/jbc.M114.593343
- Matosevic S, Paegel BM. Layer-by-layer cell membrane assembly. [Article]. *Nat Chem*. 2013; 5(11):958–963.10.1038/nchem.1765 [PubMed: 24153375]
- Merkle D, Kahya N, Schwille P. Reconstitution and Anchoring of Cytoskeleton inside Giant Unilamellar Vesicles. *Chem Bio Chem*. 2008; 9(16):2673–2681.10.1002/cbic.200800340

- Mikami S, Kobayashi T, Imataka H. Cell-free protein synthesis systems with extracts from cultured human cells. *Methods Mol Biol.* 2010; 607:43–52.10.1007/978-1-60327-331-2\_5 [PubMed: 20204847]
- Mikami S, Kobayashi T, Machida K, Masutani M, Yokoyama S, Imataka H. N-terminally truncated GADD34 proteins are convenient translation enhancers in a human cell-derived in vitro protein synthesis system. [Research Support, Non-U.S. Gov't]. *Biotechnol Lett.* 2010; 32(7):897–902.10.1007/s10529-010-0251-7 [PubMed: 20349333]
- Mikami S, Kobayashi T, Masutani M, Yokoyama S, Imataka H. A human cell-derived in vitro coupled transcription/translation system optimized for production of recombinant proteins. [Research Support, Non-U.S. Gov't]. *Protein Expr Purif.* 2008; 62(2):190–198.10.1016/j.pep.2008.09.002 [PubMed: 18814849]
- Mikami S, Kobayashi T, Yokoyama S, Imataka H. A hybridoma-based in vitro translation system that efficiently synthesizes glycoproteins. [Research Support, Non-U.S. Gov't]. *J Biotechnol.* 2006; 127(1):65–78.10.1016/j.jbiotec.2006.06.018 [PubMed: 16889861]
- Mikami S, Masutani M, Sonenberg N, Yokoyama S, Imataka H. An efficient mammalian cell-free translation system supplemented with translation factors. [Comparative Study Research Support, Non-U.S. Gov't]. *Protein Expr Purif.* 2006; 46(2):348–357.10.1016/j.pep.2005.09.021 [PubMed: 16289705]
- Nishimura K, Suzuki H, Toyota T, Yomo T. Size control of giant unilamellar vesicles prepared from inverted emulsion droplets. *Journal of colloid and interface science.* 2012; 376(1):119–125.10.1016/j.jcis.2012.02.029 [PubMed: 22444482]
- Noireaux V, Libchaber A. A vesicle bioreactor as a step toward an artificial cell assembly. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A.* 2004; 101(51):17669–17674.10.1073/pnas.0408236101 [PubMed: 15591347]
- Osawa M, Anderson DE, Erickson HP. Reconstitution of Contractile FtsZ Rings in Liposomes. *Science.* 2008; 320(5877):792–794.10.1126/science.1154520 [PubMed: 18420899]
- Pautot S, Frisken BJ, Weitz DA. Engineering asymmetric vesicles. *Proceedings of the National Academy of Sciences of the United States of America.* 2003; 100(19):10718–10721.10.1073/pnas.1931005100 [PubMed: 12963816]
- Schwanhauser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Selbach M. Global quantification of mammalian gene expression control. [Research Support, Non-U.S. Gov't]. *Nature.* 2011; 473(7347):337–342.10.1038/nature10098 [PubMed: 21593866]
- Shum HC, Lee D, Yoon I, Kodger T, Weitz DA. Double Emulsion Templated Monodisperse Phospholipid Vesicles. *Langmuir.* 2008; 24(15):7651–7653.10.1021/la801833a [PubMed: 18613709]
- Tawfik DS, Griffiths AD. Man-made cell-like compartments for molecular evolution. [Research Support, Non-U.S. Gov't]. *Nat Biotechnol.* 1998; 16(7):652–656.10.1038/nbt0798-652 [PubMed: 9661199]
- Tsai FC, Stuhmann Br, Koenderink GH. Encapsulation of Active Cytoskeletal Protein Networks in Cell-Sized Liposomes. *Langmuir.* 2011; 27(16):10061–10071.10.1021/la201604z [PubMed: 21707043]
- Utada AS, Chu LY, Fernandez-Nieves A, Link DR, Holtze C, Weitz DA. Dripping, Jetting, Drops, and Wetting: The Magic of Microfluidics. *MRS Bulletin.* 2007; 32(09):702–708.10.1557/mrs2007.145
- Utada AS, Fernandez-Nieves A, Stone HA, Weitz DA. Dripping to Jetting Transitions in Coflowing Liquid Streams. *Physical Review Letters.* 2007; 99(9):094502. [PubMed: 17931011]
- Utada AS, Lenceau E, Link DR, Kaplan PD, Stone HA, Weitz DA. Monodisperse Double Emulsions Generated from a Microcapillary Device. *Science.* 2005; 308(5721):537–541.10.1126/science.1109164 [PubMed: 15845850]
- Vincent N, Roy BZ, Jeremy G, Hanna S, Albert L. Toward an artificial cell based on gene expression in vesicles. *Phys Biol.* 2005; 2(3):P1. [PubMed: 16224117]
- Yu W, Sato K, Wakabayashi M, Nakaishi T, Ko-Mitamura EP, Shima Y, Yomo T. Synthesis of functional protein in liposome. *J Biosci Bioeng.* 2001; 92(6):590–593. [http://dx.doi.org/10.1016/S1389-1723\(01\)80322-4](http://dx.doi.org/10.1016/S1389-1723(01)80322-4). [PubMed: 16233152]

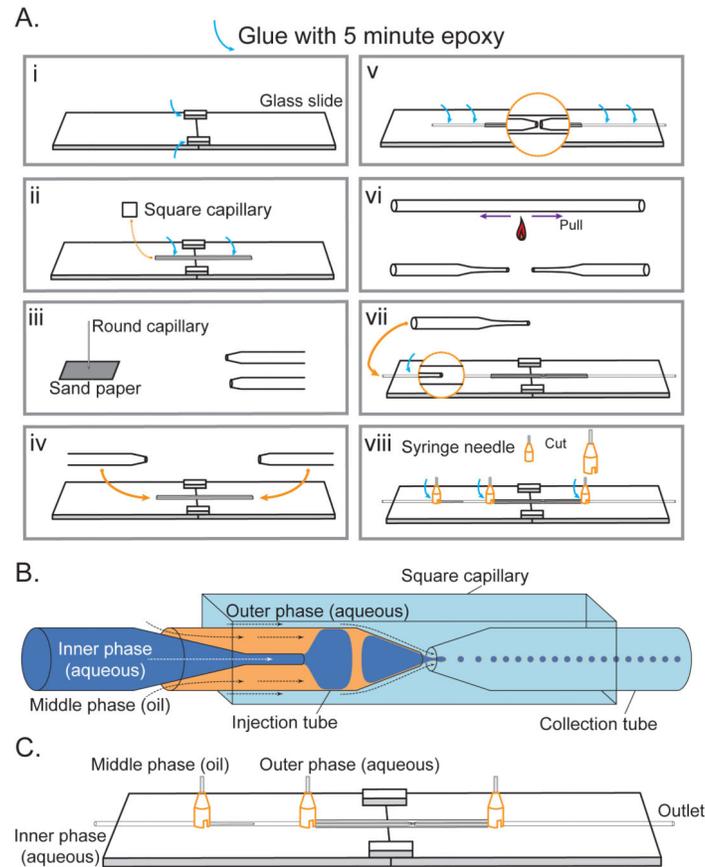


**Figure 10.1.** Schematic of how artificial cells can be engineered by combining cell-free expression technology and double emulsion template.

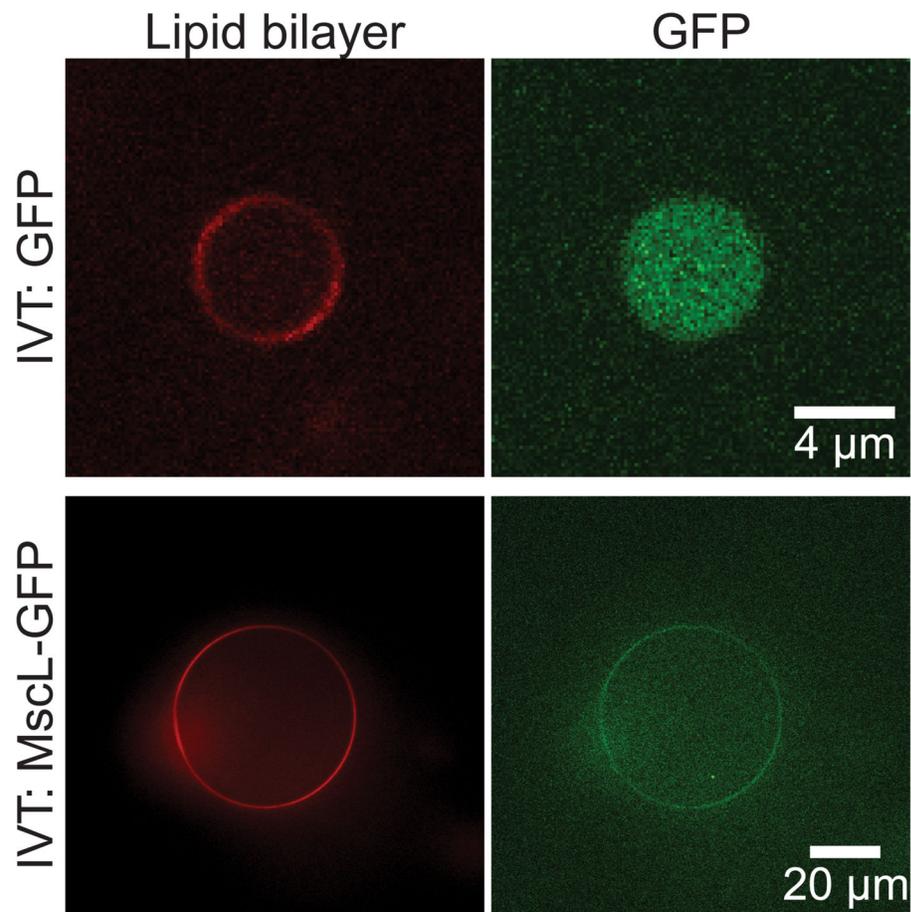


**Figure 10.2.**

(A) Expression of soluble GFP over 4 hours at 32°C measured by a plate reader ( $\lambda_{\text{ex}} = 482$  nm,  $\lambda_{\text{em}} = 512$  nm). Relative fluorescence intensity is plotted on left y-axis (normalized to 1). Concentration was determined using standard curve generated in subsection 10.1.3.1, Step 6 and is plotted on right y-axis. (B) Confocal images on lipid-coated beads incubated either with soluble GFP DNA (top row) or with GFP-MscL DNA (bottom row). SUVs were generated with Liss-Rhod-PE lipids to visualize the lipid membrane (left column). Corresponding GFP detection is shown (right column).

**Figure 10.3.**

**(A)** Step-by-step instructions on building double emulsion device. (i, ii) Square glass capillary is glued to the device platform made of two glass slides. (iii) Two tapered round capillaries are sanded to modify the diameter of the openings. (iv, v) The two round capillaries are inserted inside the square capillary and glued to the glass slide. (vi, vii) Another round capillary is pulled under frame and inserted into one of the round capillary. (viii) Syringe needles are cut and glued onto the device and serve as the inlets of the device. **(B)** The inner aqueous phase is pumped into the pulled round capillary. Middle oil phase is pumped into the coaxial region between the pulled and injection tube. Outer aqueous phase is pumped into the coaxial region between the injection tube and square capillary. Ultra-thin double emulsions will form when round capillaries are properly aligned inside the square capillary and the surfaces of glass capillaries have been modified to prevent wetting of aqueous phase. **(C)** The microtubings connect the three syringes containing inner, middle and outer phases are to the glass capillary and syringe needles in the way shown.



**Figure 10.4.** Confocal images of vesicles expressing either soluble GFP (top row) or GFP-tagged MscL (bottom row). The vesicles contained Liss-Rhod-PE lipids so lipid membrane can be visualized (left column). Corresponding GFP detection is shown (right column).