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Time-Resolved Tracking of a Minimum Gene Expression System Reconstituted in Giant Liposomes

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Synthesizing artificial cell models not only contributes to development of a new technology for crafting novel bioreactors but also facilitates understanding of the general principles of living matter. An essential step towards this end is the reconstitution of minimum gene expression systems in synthetic vesicles and monitoring their expression profiles in real time. Ideally, the gene expression systems should be constructed with defined factors (no black box), and the expression profiles of individual cell models should be analyzed independently, because genetically identical living cells sometimes show significant variability in their gene expression profiles.[1] To achieve such objectives, the capability to construct well-defined gene expression systems in artificial cell models (for example, giant liposomes; that is, lipid vesicles with diameters in the 10–100 μm range) has thus been recognized as an important experimental challenge.[2] Various methodologies to generate giant liposomes—such as natural swelling,[3] electroformation,[4] microfluidics,[5] or microjetting[6]—have been proposed. However, the capacity to encapsulate gene expression systems in giant unilamellar liposomes by these techniques remained challenging: only 10–30% of liposomes encapsulate desired biomacromolecules in the natural swelling method, for instance. Szostak and his colleagues successfully incorporated nucleotides or RNA inside model protocell compartments, but the obtained vesicles were small, with no defined shape and no phospholipid in the membrane.[7]

In contrast, it has recently been shown that phospholipid-coated water-in-oil microdroplets[8] can be used as precursors to generate giant unilamellar liposomes capable of encapsulating gene expression systems.[9] Using this principle, Libchaber et al. were able to encapsulate cell-extracted transcription/translation systems in liposomes, which maintained gene expression for about four days.[9a] However, they used a centrifugation strategy to collect liposomes, which resulted in low yields of vesicle production and a dispersion of generated liposomes in the water solution.

To overcome the above issues, we have developed a method in which a large number of individual giant liposomes encapsulating well-defined transcription/translation systems can be generated and tracked simultaneously in real time. It is based on the spontaneous transfer of water-in-oil microdroplets through an oil/water interface. Under optimized conditions, hundreds of liposomes can be generated and localized below the interface, which allows for real-time determination of their gene expression profiles by confocal microscopy. We applied the methodology to investigate the dynamics of gene expression systems reconstituted from purified components inside the generated liposomes.

Figure 1 shows the principle of our methodology. A reconstituted gene expression or translation system is assembled at 4 °C and emulsified in mineral oil containing egg yolk L-α-phosphatidylcholine phospholipid (egg PC). The emulsion is added to an oil phase containing egg PC, situated above a water phase in an observation chamber at 37 °C (Figure 1A), where transcription/translation can proceed. Within a minute, most of the droplets have spontaneously transferred though the oil/water interface to form liposomes just below the interface, which is consistent with previous observations.[9b,10] Interestingly, most generated liposomes remain attached to the interface, which enables the simultaneous characterization of a large number of liposomes by confocal microscopy (Figure 1B). For the reconstituted gene expression system we used PURESYSTEM, a commercially available cell-free transcriptional and translational system reconstituted with purified protein factors.[11] PURESYSTEM offers various advantages: 1) it is composed of purified components with a well-defined concentration, 2) it is adapted for protein synthesis in liposomes,[12] and 3) both DNA and RNA fragments can be used as templates because the system is free of RNase. To obtain a large number of stable giant liposomes that contained PURE solutions, we initially optimized the buffer conditions of "inside" and "outside" solutions (Experimental Section; see also Figure S1 in the Supporting Information). Under these conditions, a water-in-oil emulsion containing PURE solution spontaneously transferred through the oil/water interface, leading to the formation of hundreds of stable liposomes with sizes in the 1–100 μm range. Once encapsulated, the reconstituted system could effectively express proteins in the liposomes. As an example, DNA fragments coding for a recombinant green fluorescent protein (GFPuv) had been transcribed and translated after 1 h incubation at 37 °C whereas no fluorescence was observed in

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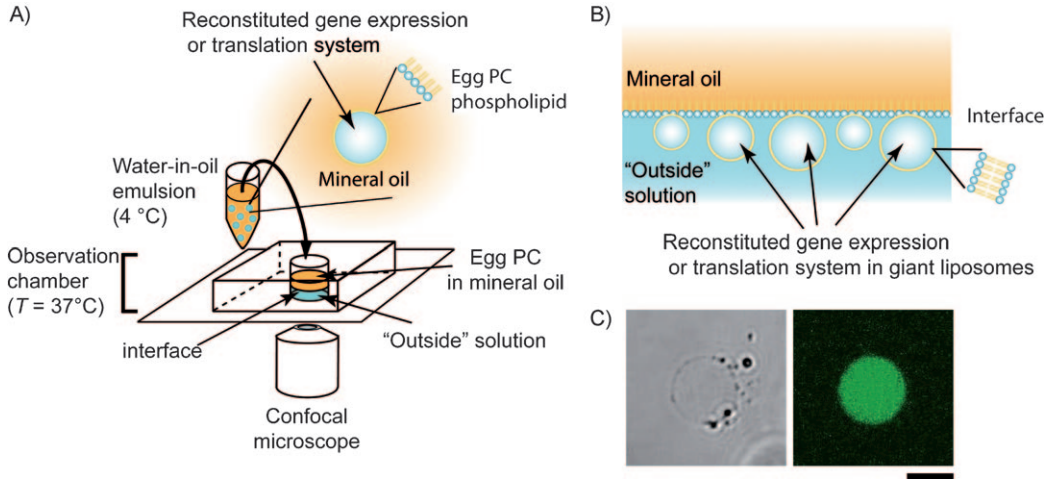

Figure 1. Experimental protocol. A) Principle of the experiment. B) Droplets spontaneously transfer through the oil/water interface to form giant liposomes encapsulating the reconstituted gene expression system. Most of the formed liposomes are immobilized just below the interface. C) Confocal transmission (left) and fluorescence (right) image of a giant liposome expressing a recombinant green fluorescent protein (GFPuv). DNA template concentration is 50 $\mu g\,mL^{-1}$. The scale bar is 20 μm.

the absence of the template DNA (Figure 1C), indicating that transcription and translation reactions had successfully proceeded in the liposomes.

We next studied the possibility of monitoring gene expression in the generated liposomes in real time. We first used DNA fragments coding for enhanced green fluorescent protein (EGFP) as templates. A large number of liposomes were obtained below the interface, and gene expression was monitored by confocal fluorescence microscopy. Figure 2 shows typical time-lapse images of EGFP expression in liposomes and shows that EGFP is expressed simultaneously in many liposomes. A weak fluorescence can be detected at ~15 min. With time, the fluorescence progressively increases to reach a plateau after 75–90 min. Notably, there is significant variability in the levels of expression in the individual liposomes, although they encapsulate the same genetic material.

To quantify the gene expression profile in each individual liposome, we developed an image analysis program that detected individual liposomes and measure their sizes and their fluorescence intensities. By encapsulating various known amounts of GFP in giant liposomes, we demonstrated that fluorescence intensity as determined by confocal microscopy is a direct measurement of GFP expression and that it can be used to quantify the absolute amount of synthesized GFP (Figure S2). Figure 3 shows the real-time gene expression profiles in many individual liposomes encapsulating DNA or RNA templates coding for different proteins (EGFP or GFPuv).

Regardless of template nature (DNA, RNA) and sequence (EGFP or GFPuv), similar features can be observed: 1) there is a significant variability in the gene expression levels between individual liposomes, 2) the average curves of individual gene expression profiles (thick red curves) have general shapes similar to those measured in bulk conditions (insets), and 3) although there is a weak tendency for larger liposomes to have higher levels of expression, there is no significant correlation between gene expression level and liposome size. The difference between the average curves for EGFP–DNA and GFPUV–DNA (Figure 3A and C) are likely attributable to the different maturation times of the two proteins. It is also interesting to compare the profiles of EGFP expression for DNA and RNA templates. The liposomes containing PURE solution and *EGFP* mRNA started to express detectable fluorescence after 10 min incubation and reached a plateau after 60 min. Conversely, the liposomes containing *EGFP* DNA started to express fluorescence after ~15 min incubation, and their intensity reached a plateau around 75–90 min. This shows that EGFP expression can be measured independently in real time for DNA and mRNA templates, which allows one to distinguish between the contributions of transcription and translation in the gene expression process. We then repeated the experiment for different concentrations of DNA (from 0.01 to 100 $\mu g\,mL^{-1}$) and mRNA templates (from 25 to 1000 nM). The general profile shapes were similar to those shown in Figure 3A and B. EGFP production increased on average with an increase in template concentration and tended to saturate at high concentrations (Figure S3 in the Supporting Information). The variability between individual gene expression profiles was observed both with DNA and with mRNA templates, indicating that variability emerges at both transcription and translation steps.

In this communication we have shown what is, to the best of our knowledge, the first real-time monitoring of gene expression in a large number of individual giant liposomes encapsulating purified genetic material. Our method is based on the spontaneous transfer of water-in-oil microdroplets that encapsulate reconstituted gene expression systems through an oil/water interface. Using this technique, we established real-time gene expression profiles from DNA and mRNA templates coding for different proteins. Strong variability among the liposomes was observed at both transcriptional and translational levels, whereas the averaged profiles of individual liposomes were similar to those measured in the bulk solution.

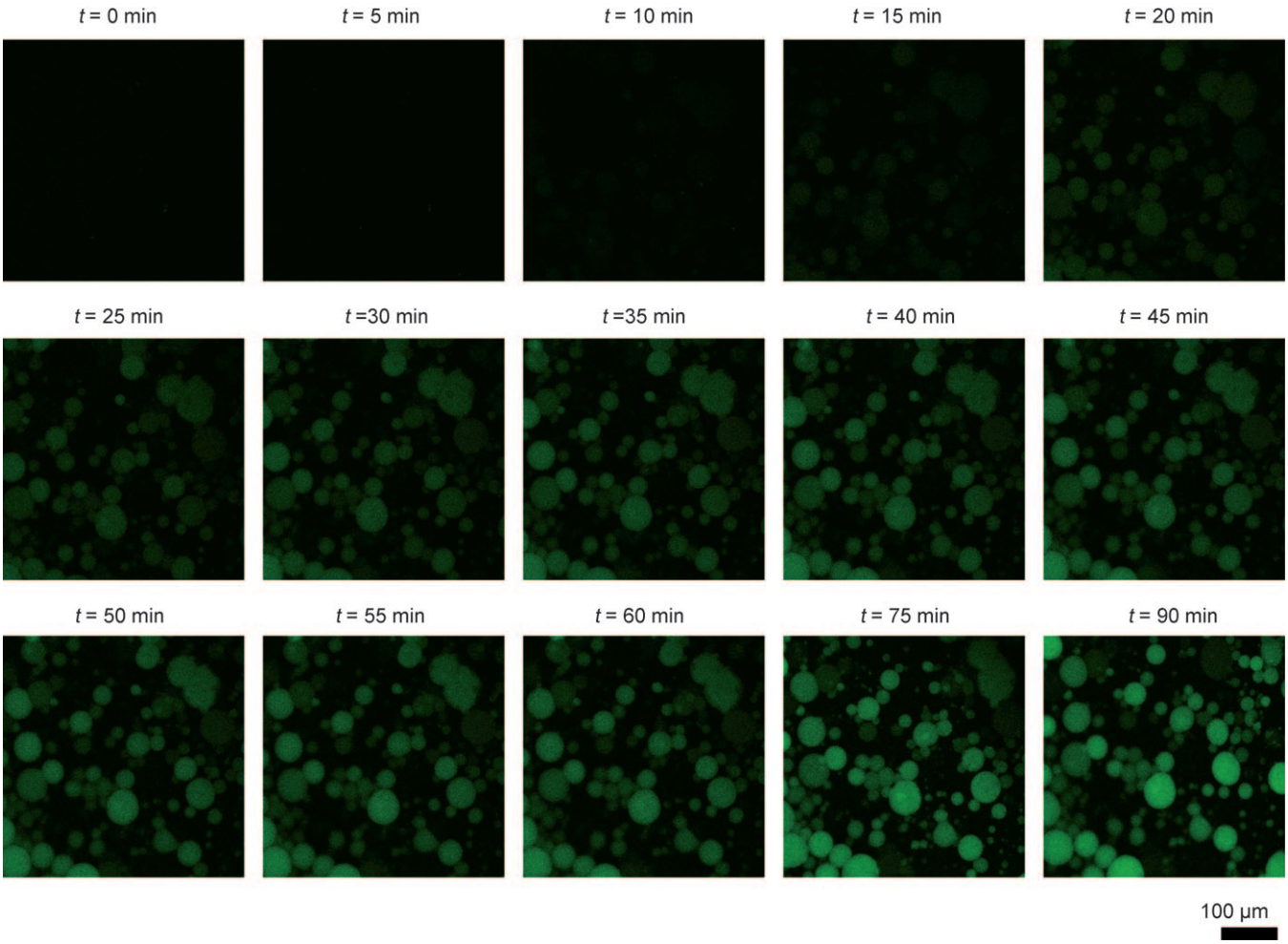

Figure 2. Time-lapse confocal fluorescence images of liposomes encapsulating the EGFP (enhanced green fluorescent protein) expression system. The DNA template concentration is 200 $\mu g\,mL^{-1}$, and the focal plane is just below the oil/water interface. $t = 0$ corresponds to the introduction of the emulsion in the observation chamber at 37 °C and also to the initiation of the reaction. Liposomes were generated within one minute after micro-droplet introduction. Overall, there is ± 1 min uncertainty in the time determination of expression in liposomes.

Experimental Section

DNA, RNA, and protein preparation: See the Supporting Information.

In vitro translation assay: In vitro translation reactions encapsulated in liposomes were carried out with PURESYSTEM classic II 96 (Post Genome Institute, Co., Ltd. Japan). PURESYSTEM is composed of solution A and solution B. We prepared solution A in house. Briefly, solution A contains Hepes/KOH (pH 7.6, 100 mM), L-glutamic acid monopotassium salt (200 mM), spermidine (4 mM), $Mg(OAc)_2$ (26 mM), dithiothreitol (DTT; 2 mM), tRNA mix (112 OD mL^{-1}), 10-formyl-5,6,7,8-tetrahydrofolic acid (20 $\mu g\,mL^{-1}$), ATP (4 mM), GTP (4 mM), CTP (2 mM), UTP (2 mM), creatine phosphate (40 mM), and the 20 standard proteinogenic amino acids (0.6 mM). Solution B contains ribosome and various protein factors, such as T7 RNA polymerase and IF 1–3, EF-G, EF-Tu, EF-Ts, RF 1–3, and RRF.

Preparation of phospholipid-containing mineral oil: An L-α-phosphatidylcholine (egg PC, Avanti Polar Lipids) solution (10 mM in chloroform/methanol 2:1, *v/v*) was poured into a glass test tube. The organic solvent was then evaporated under nitrogen flow and dried under vacuum for 10 min to leave a thin film at the bottom of the test tube. Mineral oil (500 μL, Nacalai Tesque, Inc., Japan) was then added onto the film in the test tube prior to ultrasonication for 60 min at 50 °C. After ultrasonication, the solution was immediately mixed by vortex for 20 s. For all experiments, the phospholipid solution was used within a few hours. The final phospholipid concentration in mineral oil was 0.75 mM.

Preparation and observation of the oil/water interface: The observation chamber was made of poly(dimethylsiloxane) (PDMS) with a cylindrical hole (about 4 mm in diameter and about 5 mm depth) bound to a clean microscope cover glass slide. The "outside" solution (10 μL) was spread at the bottom of the chamber to make a thin layer and topped with mineral oil (10 μL) containing egg PC (0.75 mM). The chamber was placed on the temperature-controlled stage of an inverted Axiovert 100 microscope (Zeiss) fitted with a LSM 510 module for confocal microscopy (Zeiss) and a thermocontrol unit (Tokai Hit, Japan) and was incubated for ~10 min at 37 °C prior to initiation of the translation reaction.

Preparation of liposomes with gene expression reactants: "Inside" PURESYSTEM solution (2.5 μL) was emulsified in mineral oil (50 μL) by pipetting up and down. The obtained W/O emulsion was added right after its preparation into the observation chamber above the first two layers. Within one minute, the W/O droplets had spontaneously transferred to form liposomes at the oil/water

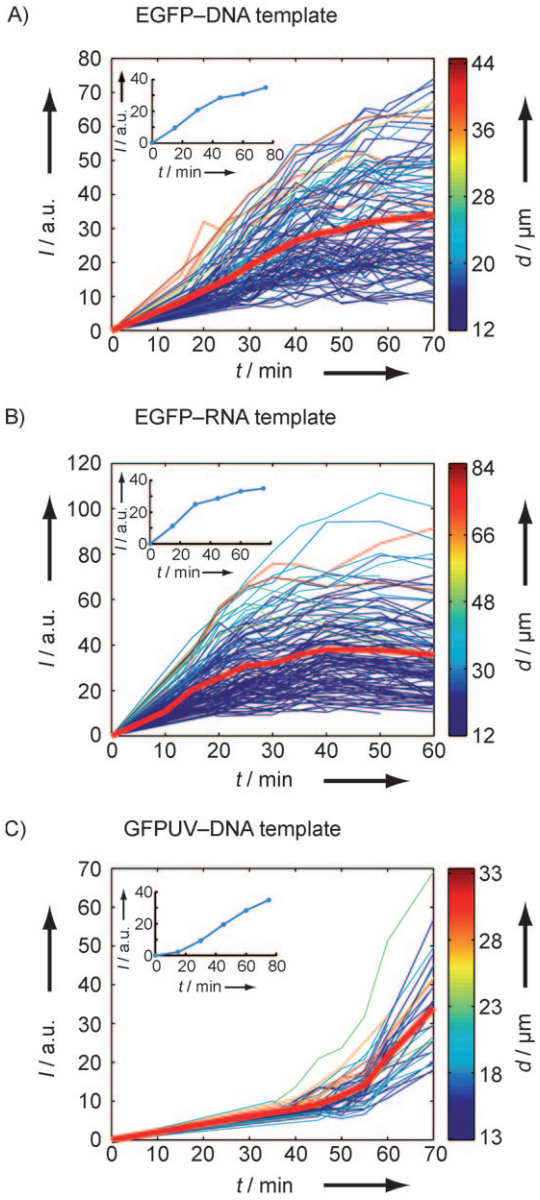

Figure 3. Fluorescence intensities of proteins expressed in liposomes from: A) DNA coding for EGFP (100 μg mL^{-1}), B) RNA coding for EGFP (1 μM), and C) DNA coding for GFPuv (100 μg mL^{-1}) as a function of time. Each thin line corresponds to an individual liposome with a color indicating the liposome diameter (color bar). The thick red lines correspond to the averages of individual curves. Insets: fluorescence intensities as a function of time measured in bulk conditions.

interface. The PURESYSTEM solution was kept on ice until emulsification, and gene expression was initiated in the observation chamber maintained at 37 °C. To generate stable liposomes at the oil/water interface, "outside" and "inside" solutions were composed as follows. 1) "Outside" solution: Solution A (5 μL), deionized water (4.6 μL), pure mix (0.4 μL). 2) "Inside" solution: Solution A (5 μL), deionized water (2 μL), solution B (2 μL), template DNA or RNA (1 μL).

Image analysis: Custom-built image analysis software was created on a Matlab platform. The liposome detection was based on shape analysis of intensity profiles in each microscopy image. The program can follow movement of each liposome (due to the fluctuations of the interface) from one picture to the other by a test in liposome position and size. A few liposomes (aggregated, nonspherical, etc…) were not detected by this technique, but all detected liposomes effectively correspond to real liposomes. We successfully detected ~80% of the generated liposomes.

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Keywords: DNA · GFP expression · giant liposomes · RNA · translation

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