In vitro gene expression within membrane-free coacervate protocells

T-Y. Dora Tang,[a] Dirk van Swaay,[b] Andrew deMello,[b] J. L Ross Anderson,[c] and Stephen Mann[a] *

Supporting Information

Methods

Coacervate-mediated cell-free gene expression of mCherry

Preparation of reaction mixtures: Cell-free gene expression of functional mCherry was performed at pH 8 within the molecularly crowded environment of a CM-dextran/polylysine (PLys) coacervate prepared as a bulk phase or as an aqueous suspension of micro-droplets. The coacervate was typically prepared by mixing equimolar aqueous solutions of CM-dextran and PLys at pH 8 (final [CM-dextran monomer] : [PLs monomer] molar ratio of approximately of 1 : 0.5) to produce a turbid suspension of micro-droplets. The dispersion was centrifuged at 5000 g for at least 10 min to generate two distinct liquid phases; a denser CM-dextran/PLys coacervate bulk phase and a polyelectrolyte-depleted supernatant. The supernatant was removed from the bulk phase by pipetting, to leave the bulk coacervate phase, which was used as a molecularly crowded medium for the in vitro gene expression of mCherry. An aliquot (typically 7 μL) of the pEXP5-NT/mCherry plasmid-containing S30 cell-free reaction mixture (prepared as described below) was added to the bulk coacervate phase at an IVGES : coacervate volume ratio of 0.75 : 1. The sample was mixed thoroughly by inverting the microfuge tube and tapping the side of the vial, then reinverting and tapping the vial on the surface until all the reaction mixture had returned to the bottom of the microfuge tube, and kept on ice until the start of the experiment. The reaction mixture was shaken continuously at 180 rpm at a constant temperature of 18^{o}C.

The above conditions were optimal for the gene expression system used. Other experiments were run in buffer (pH 8) at temperatures of 25 or 36^{o}C. Under these conditions, the initial rate of protein synthesis was enhanced but the level of protein aggregation also increased.

Monitoring of gene expression: The kinetics of cell-free gene expression in a CM-dextran/PLys coacervate were determined by confocal fluorescence microscopy. A set of individual reaction mixtures containing a 1 : 0.75 [CM-dextran/PLys] : IVGES volume ratio was incubated as described above at 18^{o}C and stirred at 180 rpm. Individual reaction mixtures were then removed from the incubator every 2 h over a period of 24 h, and in each case immediately cooled to -20 ^{o}C to quench protein synthesis. The samples were warmed to room temperature and the coacervate disassembled by addition of a high ionic strength buffer (typically, 9 μL buffer was added to 9 μL of the reaction mixture, final concentrations 1.5 M NaCl, 250 mM $NaHPO_4$, and 100 mM imidazole) that also contained Kanamycin (final concentration 14.3 mg/mL, a ribosome-binding antibiotic) to ensure that protein expression was terminated after coacervate disassembly. The solutions were centrifuged, and 2 μL of the mCherry-containing supernatant was loaded into a capillary microscope slide container. The entrance was sealed with vacuum grease to avoid sample evaporation, and the sample taken at each time point imaged using a Leica SP8 AOBS confocal system attached to a Leica

DMI 6000 inverted microscope. The sample was excited with an orange HeNe (594 nm) laser and imaged at 10x magnification. Spectral scans were collected using hybrid detectors from 601 to 750 nm with a bandwidth of 5 nm and increment of 3 nm.

Values of the fluorescence intensity at 610 nm from confocal microscopy images were used to assess the time-dependent change in mCherry concentrations associated with gene expression in the bulk coacervate phase. The fluorescence intensities were converted to approximate protein concentrations from a calibration curve determined from known concentrations of mCherry obtained by IVGES expression and using the same confocal microscopy settings. Protein concentrations obtained from the fluorescence measurements were corrected to account for dilution factors arising from salt-induced disassembly of the coacervate phase during the analytical procedure. Each data point was an average of 15 experiments. Fits to the measured data gave R^2 values greater than 0.98. The data were averaged using the "Average multiple curve functions" in ORIGIN 9 and fitted to a logistic function:

$$[mCherry] = \frac{K}{1 + e^{-B\,(t - t_{1/2})}} \qquad (1)$$

where *K* is the maximum protein concentration, B is the maximum growth rate and $t_{1/2}$ is the time of maximum growth rate; ie. the time where half the maximum protein concentration is reached.

Control experiments: The *in vitro* cell-free gene expression of mCherry from pEXP5-NT/mCherry was also undertaken as described above but in the presence of CM-dextran, PDDA, PLys or ATP. 16 µL of the IVGES was added to aqueous solutions of the polyelectrolytes or mononucleotide to give a final concentration of 100 mM CM-dextran, 100 mM PDDA, 100 mM PLys or 30 mM ATP in HEPES buffer (final concentration, 0.02 M). Monitoring of the kinetics of gene expression was undertaken as above using confocal fluorescence spectroscopy.

mCherry synthesis in individual coacervate micro-droplets

A PDMS microfluidic flow-focusing channel device coupled to a chip-to-world connector [see D. van Swaay, J-P. Machler, C. Stanley, A. deMello *Lab Chip*, **2013**, *14*, 178-181] was used to disperse a continuous stream of a homogenised IVGES/CM-dextran/PLys bulk coacervate in 0.02M HEPES into membrane-free micro-droplets in water. The device was prepared by conventional soft lithography techniques and treated with 5% 1H,1H,2H,2H-perfluorooctyltrichlorosilane (PFOS) to minimise interactions between the coacervate fluid and PDMS channels.

Formation of mCherry in the CM-dextran/PLys coacervate micro-droplets prepared by microfluidic injection was ascertained by analysis of optical and fluorescence microscopy

images of dispersions mounted into 96-well plates. Images were taken from at least four different wells using an inverted microscope (Eclipse Ti-U, Nikon, Japan) with wide-field camera (CoolSNAP HQ2, Photometrics, USA) after droplet collection (1 hr) and after 16 hrs of incubation. Exposure and acquisition were controlled using Micro Manager software [see A. Edelstein, N. Amodaj, K. Hoover, R. Vale, N. Stuurman. UNIT 14.20 Computer Control of Microscopes Using μManager. *Current Protocols in Molecular Biology*. Published Online: 1 OCT 2010. DOI: 10.1002/0471142727.mb1420s92].

Fluorescence images were obtained using a 559/34 nm excitation and 630/69 nm emission filter set. *Volocity* was then used to analyse each image to determine the mean fluorescence intensity (*I*) of an individual droplet as a function of its surface area (*P*). For each image, a background intensity was obtained and removed from the mean intensity for each droplet. *Origin* was used to fit a linear regression to the data and the gradients were compared to CM-dextran/PLys coacervate droplets containing IVGES in the presence of the pEXT5-NT/CALML3 plasmid.

Extraction, isolatation and purification of expressed mCherry from coacervate media

Disassembly of the CM-dextran/PLys coacervate matrix at pH 8 was achieved by addition of a high ionic strength buffer (typically 9 μL buffer was added to 9 μL of the reaction mixture, final concentrations 1.5 M NaCl, 250 mM $NaHPO_4$, and 100 mM imidazole). Kanamycin (final concentration 14.3 mg/mL, a ribosome-binding antibiotic) was also added to the buffered solution to ensure that protein expression was terminated after coacervate disassembly. The solutions were then vortexed for 3 sec, followed by room temperature centrifugation for 5 min at 1300 rpm to separate any insoluble components from the soluble mCherry-containing supernatant. The supernatant was removed and incubated overnight at 8^{o}C with agarose beads coupled with a red fluorescent protein (RFP) binding protein (ChromoTek Gmbh, Germany) in order to trap mCherry onto the bead. (To prepare the beads, 3 μL of as-received beads were incubated in 80 μL of Trap buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) and washed at least three times by centrifugation at 2000 rpm for 3 mins at 4^{o}C, followed by removal of 65 μL of buffer). After incubation with the mCherry containing supernatant, the mCherry-trapped beads were washed at least 5 times with buffer, and their fluorescence intensities determined using confocal fluorescence microscopy at excitation and emission wavelengths of 594 and 601-750 nm, respectively, with a 5 nm bandwidth and at 3 nm increments. Purified mCherry was removed from the trap beads by incubating the beads at 96^{o}C for 10 mins with Sample application buffer (62.5 mM Tris-HCl pH 7, 2.5 % SDS, 0.002 % bromophenol Blue, 0.7 M β-mercaptoethanol, 10% glycerol), followed by centrifugation for 5 mins at 2000 rpm. The mCherry-containing supernatant was removed and characterised using Weston Blot analysis.

General methods

Cell-free gene expression of mCherry. Transcription and translation of mCherry from pEXP5-NT/mCherry was undertaken using the Expressway Cell-Free *E. co*li Expression System (Invitrogen) comprising S30 *E. coli* extract, reaction buffer, proprietary T7 enzyme mix and feed buffer (including magnesium acetate, potassium glutamate, cAMP, NAD) The kit and pEXP5-NT/mCherry plasmid were mixed under sterile conditions and on ice (typically, 20 µL S30 *E. coli* extract, 20 µL reaction buffer, 25 µL feed buffer, 1 µL proprietary T7 enzyme mix, 4.5 µL 50 mM amino acid solution, and 1 µg of pEXP5-NT/mCherry), and used according to the manufacturer's instructions unless otherwise stated. The *in vitro* gene expression system (IVGES) was mixed by gently tapping the side of the microfuge tube and then ensuring that all of the reaction mixture had returned to the bottom of the tube.

Construction of plasmid pEXP5-NT/mCherry. The mCherry gene was amplified from pBAD/mCherry and inserted into pEXP5-NT/CALML3 using ligase-independent cloning [J. Chiu, P. E. March, R. Lee, D. Tillett, *Nucleic Acids Res* **2004**, *32*, e174-e174] to yield the plasmid pEXP5-NT/mCherry. Primers were designed to facilitate replacement of the CALML3 gene with the mCherry insert whilst retaining the TEV-cleavable N-terminal hexahistidine tag. The plasmid retained the ampicillin resistance gene, T7 promoter site, ribosome binding site, and the pUC origin of replication sequence. pEXP-NT/mCherry was sequenced (Scheme 1, Supporting Information) and transformed into *E. coli* BL21 competent cells for amplification. The BL21 cells were isolated by centrifugation and smeared onto an agar plate containing the antibiotic, carbenicillin and incubated overnight at 37^{o}C. A single colony was removed from the agar and incubated in Luria Bertani (LB) broth with carbenicillin overnight at 37^{o}C. The cells were harvested and the plasmid was extracted from the *E. coli* cells and purified using MINIPREP® (Qiagen, UK) resulting in a final plasmid concentration of 219 ng /µL.

Western blot analysis: Purified mCherry was obtained from cell free gene expression in a bulk CM-dextran/PLys coacervate phase by extraction from Trap beads (see Methods in main text) to produce a supernatant that was characterised using Western Blot analysis. Typically, 10 µL of supernatant and sample application buffer was loaded into Nupage Sep Tris-glycine gels 4-15 wt% (Fisher) and separated based on protein molecular weight using a constant voltage of 200 mV for 35 minutes. The protein was transferred from the gel in transfer buffer (containing 390 mM Glycine, 478 mM Tris-Base, 13mM SDS, 20 % methanol) onto a methanol-activated PVDF membrane (Millipore) at a constant current of 45 mA for 1 hour. The PVDF membrane was blocked using skimmed milk for 1.5 h at room temperature and then incubated with monoclonal anti-polyhistidine-peroxidase antibodies (0.5-1.1 µg/ml) (Sigma Aldrich) for 1 h at room temperature. The PVDF membrane was then washed at least 5 tmes with PBS-Tween (0.1%) with 20 min shaking between each rinse, and then incubated in a peroxide/luminol solution (ECL Prime, Amersham Lifescience) for 3 min prior to film exposure and development.

*Expression of pBAD/mCherry in E. coli: m*Cherry was expressed from the pBAD/MCherry plasmid (gift from Dafydd Jones, University of Cardiff) by transforming 1 µL of plasmid DNA into 50 µL of Top10 competent cells. [J. T. Hammill, S. Miyake-Stoner, J. L. Hazen, J.C.

Jackson, R.A. Mehl, *Nature protocals* 2007, **2,** 2601-2607]. The vector was added to the competent cells in ice and incubated for 30 min, heat shocked at 42 ^{o}C for 30 sec and then placed in ice for 2 min. 1 mL of SOC media was added to the cells and incubated for 1 hour at 37 ^{o}C with shaking at 180 rpm. The cells were rescued and plated onto LB agar/carbenicillin medium and left at 37 ^{o}C overnight. A single colony was selected and added to 6 mL LB broth media and incubated at 37 ^{o}C with 250 rpm shaking until the OD at 600 nm was greater than 1 (approximately 12 hours). The saturated cells were then added to pre-warmed media (1 lL, 37^{o}C) with 1 mL of 50 µg/mL of carbenicillin, and incubated at 37^{o}C at 250 rpm. 0.5 g of arabinose (Sigma Aldrich) was added the cell culture once the OD was 0.6, and then further incubated at 37^{o}C and 250 rpm for 30 hr. The cells were harvested at 4^{o}C and 4000 g for 25 min. The cells were then lysed in ice by ultrasonication after resuspension in 30 mL of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml DNASE 1 and 10 mM $CaCl_2$) and 1 mL of 100x PMSF and then centrifuged at 4^{o}C for 30 min and 20 000g. The protein was purified by loading onto a Ni-NTA column, washing the column with wash buffer and then collecting the protein by rinsing the column with buffer containing 300 mM NaCl, 50 mM Na_3PO_4, 250 mM imidazole at a concentration of approximately 37 µM.

mCherry characterisation: Purified protein from *E. coli* was characterised using fluorescence spectroscopy (Fluormax 4, Horiba scientific). Typically the protein was diluted to 1.5 µM with MiliQ water and 200 µL was loaded into a quartz cuvette. The sample was excited at 587 nm with a 5 nm bandpass. The emission was collected at right angles to the excitation between 600 and 750 nm with a 1 nm increment.

Sequence for the mCherry gene

ATGTCTGGTTCTCATCATCATCATCATCATGGTAGCAGCGGCGAAAACCTGTATTTTCAGTCCAT
GGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCA
CATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCT
ACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGG
GACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCC
CCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGA
CGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGT
GAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTG
GGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGA
GGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGA
AGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGA
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Supporting Information: Figures

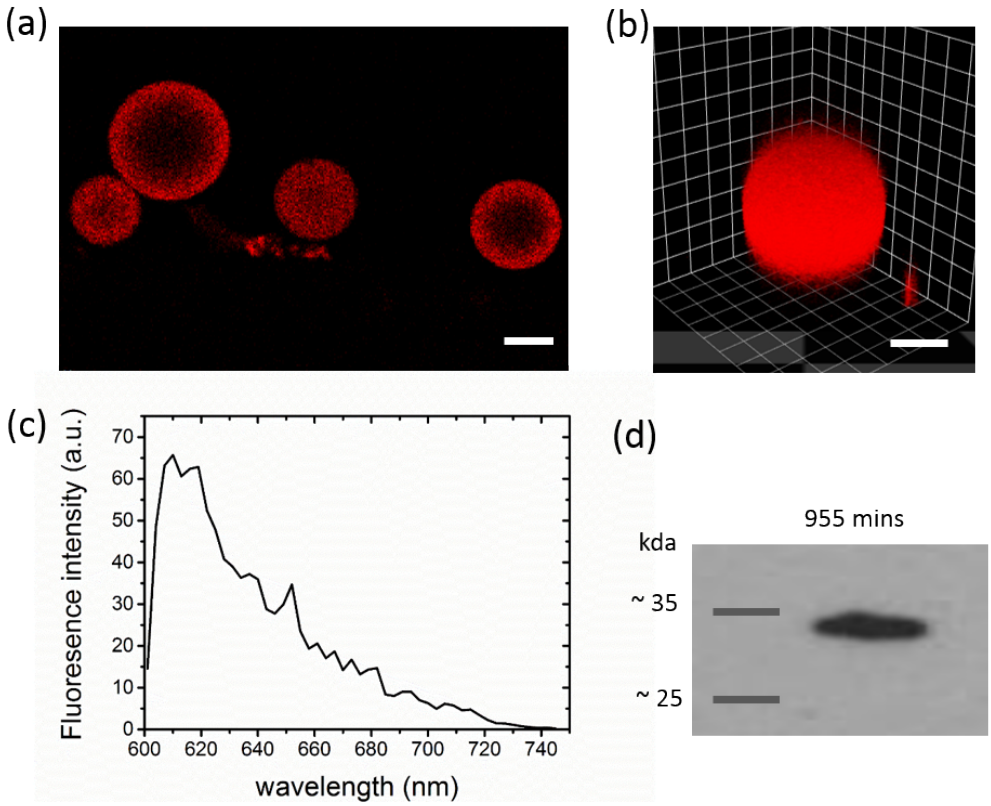

Figure S1. (**a-d**) Extraction and characterization of coacervate-mediated expression of mCherry. (**a**) Confocal microscopy image showing red fluorescence associated with binding of expressed mCherry on antibody-functionalized agarose trap beads; scale bar = 50 µm. (**b**) Stacked xyz confocal image of a single trap bead after mCherry extraction showing homogeneous distribution of bound mCherry; scale bar = 50 µm. (**c**) Fluorescence spectra obtained from a single trap bead (λ_{exc} = 594 nm, λ_{exc} = 601-750 nm) showing a characteristic mCherry peak at 610 nm. (**d**) Western Blot showing a single band at approximate 35 kDa (right lane), characteristic of mCherry with a TEV cleavable hexahistidine tag; the sample was removed from the trap beads by incubating the beads at 96^{o}C for 10 min with sample application buffer.

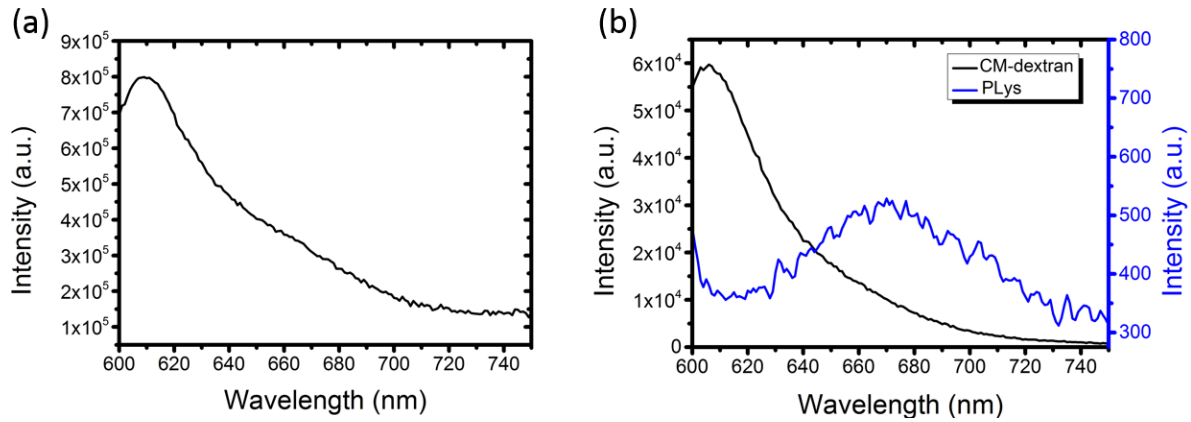

Figure S2. Fluorescence emission spectra (excitation at 587 nm) for cell-free gene expression of mCherry at 18°C for 6.5 hrs in aqueous solutions of (**a**) HEPES (0.02 M), and (**b**) CM-dextran (100 mM, black) or polylysine (100 mM, blue). Note the presence of a 610 nm peak confirming mCherry synthesis and folding in the buffer and CM-dextran controls.