Supporting Information

Construction of multiphasic membraneless organelle towards spontaneous spatial segregation and directional flow of biochemical reactions

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Supplementary Methods

Synthesis of HD-IPH with Rhodamine B labeling

For fluorophore labelling of HD, two-step one-pot synthesis was performed, where fluorophores were first reacted with Dex-VS via thiol-Michael addition reaction, followed by addition of peptides. For this purpose, fluorophore with thiol modification, RhoB-PEG1000-SH was used. All steps of the protocol were held under minimal light condition. 40kDa Dex-VS (DM_{VS} 92%) was dissolved in N2 pre-treated 3M NaCl solution at 2mg/mL. Under continuous stirring, RhoB-PEG1000-SH was slowly added to Dex-VS at 1.25x molar excess of final HD-IPH. First 10 mins, the reaction was held under N2 flow and at pH 5.5 to prevent any potential disulfide bonds. Then the pH was adjusted to 6.0 and the reaction was kept for 30 mins under continuous stirring under N2 flow. pH was then readjusted to pH 5.5 to slow down the reaction to add 'sticker' peptides. Total molarity of peptides added were at 1.25x excess to VS of Dex-VS, while peptide sequences were added at specific molar feed ratio to reach desired Y/R of ~ 1.03. To initiate the reaction, pH was readjusted back to pH 6.0 and the reagents were left to react for 24 h under continuous stirring. Materials were then dialysed for 5 days in 12-14 kDa dialysis tubing against ultrapure water, protected from light. After dialysis RhoB-labelled HD was lyophilized and stored at −20 °.

The sample was characterized with 1H NMR and conjugation ratio (F/P) was quantified based on absorbance (at 600 nm) and fluorescence measurements (excitation/emission = 491/516 nm) of the conjugate.

Aspect ratio quantification

Time lapse of the fusion events were analysed in Fiji software, following provided protocol[54]. 10 frames prior and after fusion event were used to track the aspect ratio (AR) change over the course of droplets fusion. AR here is defined as the ratio of major and minor axis of two droplets, where prior to coalescence AR of two-droplets in close proximity (dimer setting) is approximated to 2. Further coalescence of the two droplets leads to the collapse into one sphere with AR equal to 1.

T7 polymerase fluorophore labelling

T7 polymerase (10 U/µL) was prepared in PBS buffer pH 8.5 at 98 µL volume, followed by addition of 2 µL of FITC stock (1 mg/mL). Reaction solution was vortexed immediately and left to react for 40 mins, at 21 ^{o}C under continuous stirring. To remove unconjugated FITC molecules, solution was passed through Amicon Ultra 2 mL centrifugal filter (MWCO 10 kDa) two times.

Ribosome fluorophore labelling

70S E. coli ribosome was suspended at 1 µg/µL in labelling buffer consisting of 0.1 M NaHCO3 (pH 8), 10mM $MgCl_2$. FITC stock prepared in DMF was added to the ribosomes at 0.1 µg/µL bringing the reaction mixture to 200 µL. Reaction solution was mixed immediately after addition of FITC and placed at 37^{o}C for 60 min, wrapped in aluminium foil. To remove excess of fluorophores and keep the ribosomes intact, the sucrose cushion ultracentrifugation method was used[1]. 1.1 M sucrose solution was added to the bottom of centrifuge tube followed by FITC-ribosome mixture without mixing the two layers. The tube was centrifuged at 20,000xg for 60 min at 4^{o}C, after which supernatant was removed and the ribosome containing sucrose layer was redispersed in PBS buffer and washed for 2 times by centrifuging at 15,000xg for 10 min to pellet down the ribosomes. Pellets were then dissolved in the original storage buffer (20 mM HEPES-KOH (pH 7.6), 10 mM magnesium acetate, 30 mM ammonium acetate, 4 mM 2-mercaptoethanol). Concentration of labelled ribosomes was quantified using NanoVue spectrophotometer.

Plasmid construction and extraction

Plasmid pET29/GFP(+36) was purchased from Addgene in the form of agar stab. Plasmid contains T7 promoter sequence and ribosome-binding site (Shine-Delgarno sequence (SD)) following PURExpress

system plasmid design protocol[2]. Bacteria from the stab culture was streaked onto Luria Bertani (LB) agar plate with ampicillin and incubated for 16 h at 37°C. Single colony was isolated from the agar and incubated in liquid LB with ampicillin for 12 h at 37°C under continuous shaking (200 rpm). After sufficient growth was reached, inoculated liquid culture was harvested and bacterial pellets were used to extract and purify the plasmid DNA using Qiagen Plasmid Midi kit. Plasmid pellets were resuspended in TE buffer (pH 8.0) and final plasmid concentration was checked by NanoVue Spectrophotometer to be 380 ng/ µL.

Supplementary Figures

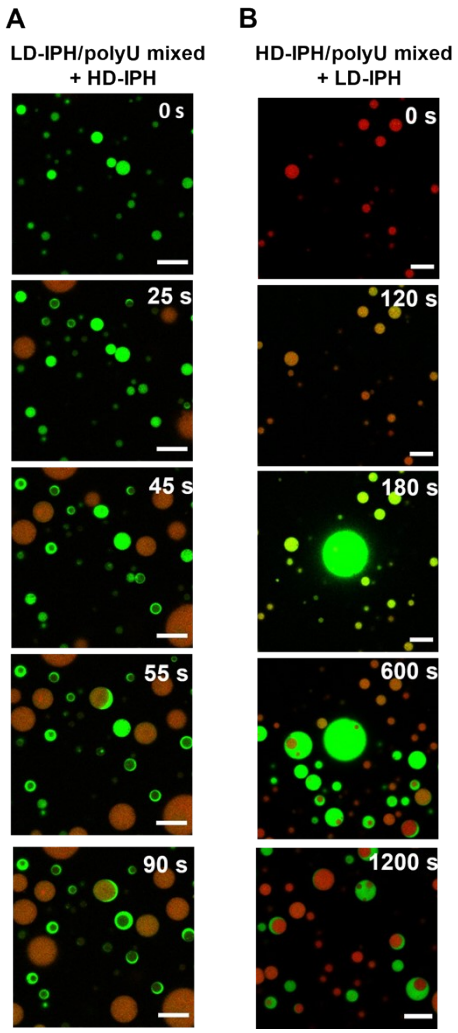


Figure S1. Mixing sequence of multiphase droplet components. Confocal fluorescent images correspond droplets structuration over time under different mixing protocol: **(A)** HD-IPH added to the mixture of LD-IPH and polyU RNA, **(B)** LD-IPH added to the mixture containing HD-IPH and polyU RNA. Green colour corresponds to FITC-labelled LD-IPH constructs and red – RhoB-labelled HD-IPH. Scale bar = 10µm

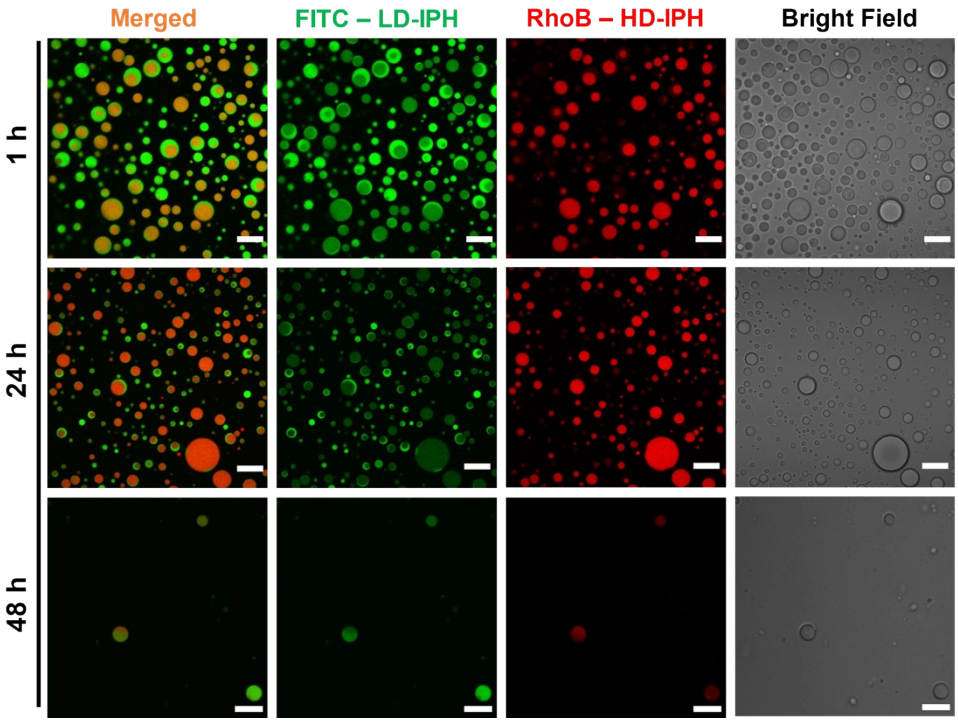


Figure S2. **Stability of LD-IPH/HD-IPH/polyU multiphasic droplet over time.** Droplets conditions tested at different time-points under fluorescent confocal microscope. Scale bar = 20 µm.

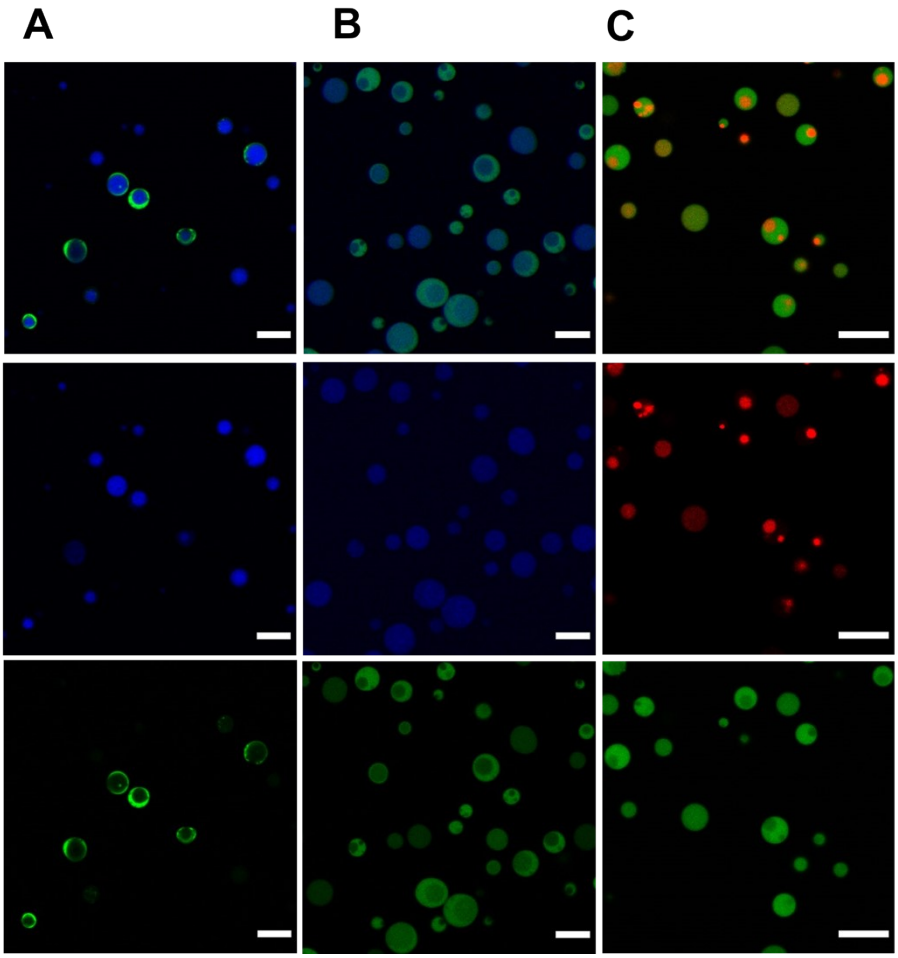


Figure S3. Multiphase droplets with different RNAs. Internal structuration of LD- and HD-IPH droplets in the presence of **(A)** homopolymeric polyA RNA (2,800-nt long), **(B)** tRNA molecules (78-nt long). Green corresponds to FITC-labelled LD-IPH constructs, and blue represents methylene blue-stained RNA molecules; **(C)** Cy5-labelled ssRNA (47-nt long).

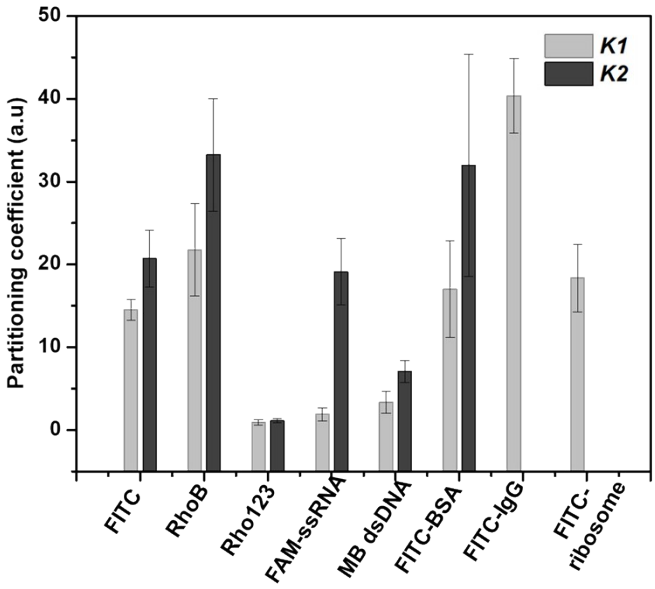


Figure S 4. Partitioning coefficients of client molecules in multiphase droplet. Partitioning coefficients estimated from fluorescent confocal microscope images, as the ratio of mean fluorescent intensities inside either the outer (I_o) or inner (I_i) compartment of the droplet phase by the mean intensities in the external dilute phase (I_{dil}). K1 – denotes partitioning in the outer droplet phase, and K2 – partitioning in the inner droplet phase.

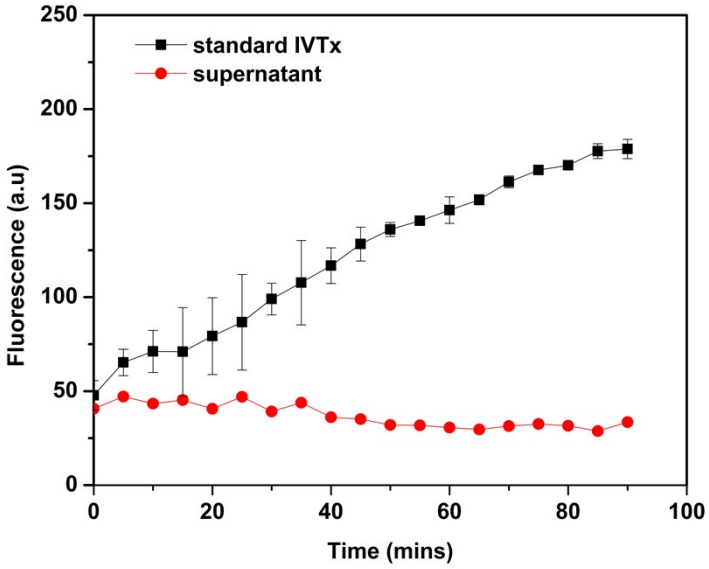


Figure S5. IVTx reaction kinetics. Plate-reader recordings of kinetics of RNA synthesis by standard IVTx (in reaction buffer) and in the supernatant (with removed droplet phase). Mean of n=3 independent readings are reported ± S.D.

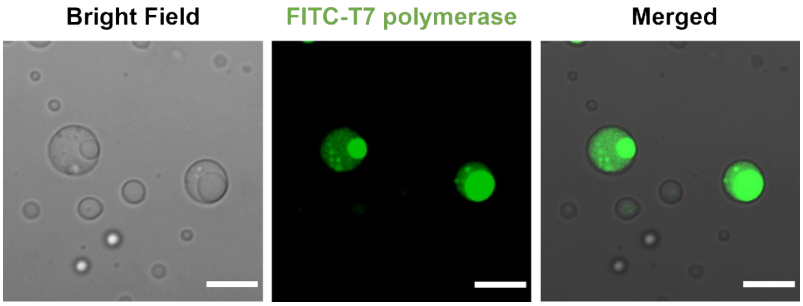


Figure S 6. Partitioning of FITC labelled T7 polymerase. Fluorescent confocal images of multiphase droplets mixed with 50 U of T7 polymerase. Scale bar = 10 µm.

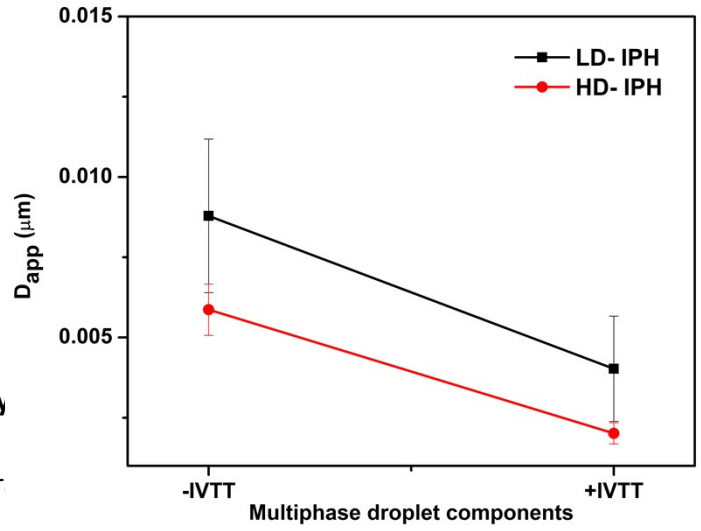
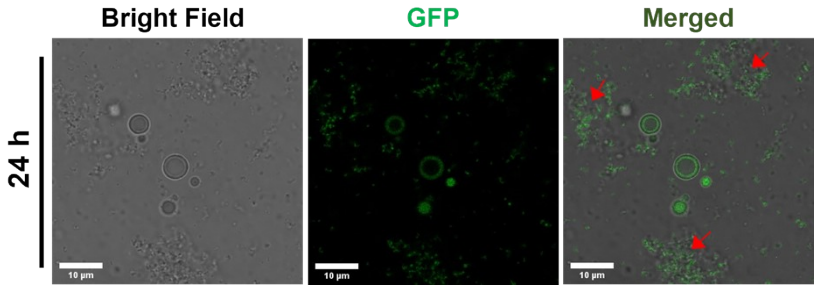


Figure S7. Mobility)-IPH and HD-IPH diffusion rate (n=3 +IVTT) of in vitro transcription and tr



References

Figure S8. IVTT reaction progression in multiphase droplets. IVTT reaction state after 24 h. Green colour corresponds to the fluorescence of expressed GFP. Red arrows indicate aggregated components within reaction solution. Scale bar = 10 µm

1. Höfig, H. *et al.* Brightness-gated two-color coincidence detection unravels two distinct mechanisms in bacterial protein translation initiation. *Commun. Biol.* **2**, 459 (2019).

2. Tuckey, C., Asahara, H., Zhou, Y. & Chong, S. Protein synthesis using a reconstituted cell-free system. *Curr. Protoc. Mol. Biol.* **108**, 16.31.1-16.31.22 (2014).