Supporting Information – Materials and Methods

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (MO, USA). All aqueous solutions were composed of de-ionised (DI) water.

Preparation of IVTT solution

For cell-free protein synthesis we used the PURExpress *in vitro* transcription and translation kit (New England Biolabs, MA, USA), and followed the supplier's recommendations for the assembly of the solution. The kit contained two tubes, each consisting of different purified cellular fractions. 5 µl of 'tube A' was added to 3.5 µl of 'tube B' followed by 125 ng of plasmid. 10 units of RNase inhibitor (RNasin, Promega, WI, USA) was also added to the mix to prevent RNA degradation. We supplemented this with an added volume, consisting of sucrose and DPhPC liposomes (Avanti Polar Lipids), formed by sonication for one hour. 3.75 µl of this solution was added to the kit, to give a final concentration of 9 mM liposomes, and 0.9 M sucrose. Two such solutions were made: one containing Dasher-GFP plasmid, the other an RFP plasmid. Plasmids coding for both these proteins were synthesised by DNA 2.0 (CA, USA), and comprised of an E. coli pJexpress 441 vector with T7 promoter and terminator sequences. Plasmid sequence consisted of: T7 promoter – Lac operator – spacer – RBS – Orf – T7 Terminator, together with AmpR. Catalogue number: FP-03-441. The mixture was gently mixed using a pipette, and was immediately used to generate multi-compartment vesicles.

Generation of multi-compartment vesicles

Two-compartment vesicles were generated using phase transfer of multiple water-in-oil droplets. First, a lipid-in-oil solution was made by sonicating DPhPC lipid in a 75:25 mixture of hexadecane and mineral oil for 60 minutes at 50° C to give a 4.7 mM solution. A water-oil column was then assembled by depositing 0.5 ml of this solution above 0.5 ml of DI water of a Lab-Tek chamber slides, 1.7 cm^2 area, 1 ml volume (Thermo Scientific, MA, USA) . This was left for two hours in order for a sufficiently packed water-oil monolayer to self-assemble at the interface. Two 0.1 µL droplets, comprising of the IVTT mix and the plasmids for the two fluorescent proteins, were then

expelled above the same location of the column. This droplet pair descended through the water-oil interface together to yield multi-compartment vesicles. Immediately after they were formed 1.5 M sucrose was injected at the bottom of the well to reduce the osmotic imbalance between the vesicle interior and exterior. Fluorescence microscopy experiments were conducted on a Nikon Eclipse TE 2000E microscope, illuminated with a mercury arc lamp, and recorded on a QICAM camera (QImaging, Surrey, Canada).

Effect of glucose and liposomes on IVTT efficiency

Experiments on the effect of sucrose and liposomes on IVTT efficiency was conducted on a 384 low-volume well plate, on a Varian Cary Eclipse fluorescent spectrophotometer (Agilent Technologies, USA). GFP emission was measured at 521 nm with excitation at 510 nm. Four different conditions tested: 0.9 M sucrose; 9 mM DPhPC liposomes; 0.9 M sucrose & 9 mM DPhPC liposomes; DI water (control). The kit was incubated for two hours at 37 °C for two hours before fluorescence intensity was recoded.