Time-resolved cryo-EM using Spotiton

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We present an approach for preparing cryo-electron microscopy (cryo-EM) grids to study short-lived molecular states. Using piezoelectric dispensing, two independent streams of ~50-pl droplets of sample are deposited within 10 ms of each other onto the surface of a nanowire EM grid, and the mixing reaction stops when the grid is vitrified in liquid ethane ~100 ms later. We demonstrate this approach for four biological systems where short-lived states are of high interest.

Cryo-EM can capture a wide variety of conformational states of macromolecules in solution. Changes in conformational states can be triggered by a variety of biological reactions; adding a ligand to an enzyme, mixing together components of a multicomponent system in solution or adding energy in the form of ATP or GTP. Conformational changes are often transient but can be trapped by vitrification at specific time points following the initiation of the reaction and imaged using electron microscopy, a process loosely referred to as ‘time-resolved cryo-EM’ when applied to the study of a conformational process occurring on the millisecond timescale.

Possibly the earliest approach to time-resolved cryo-EM was by Berriman and Unwin1, where one reactant was sprayed from an atomizer placed just above the cryogen onto a second reactant already present as a thin aqueous film on an EM grid plunging toward a cryogen at high speed. Later, White et al. described a computer-controlled, ‘spraying-mixing’ device and observed the interaction of myosin sprayed onto F-actin filaments. Although this approach can achieve time resolutions as low as 2 ms, mixing is not uniform across the EM grid, and fiducial markers are required in the sprayed solution to identify regions where mixing occurred. A third approach involved exposing crystals of bacteriorhodopsin on a computer-controlled, ‘spraying-mixing’ device and observed the interaction of myosin sprayed onto F-actin filaments1. Although this approach can achieve time resolutions as low as 2 ms, mixing is not uniform across the EM grid, and fiducial markers are required in the sprayed solution to identify regions where mixing occurred. A third approach involved exposing crystals of bacteriorhodopsin on a nanowire surface is expected. Nevertheless, as a first proof of principle, we mixed two test samples, apoferritin and 70S ribosomes. In practice, one can control the extent of sample stream overlap by adjusting either the dispenser head alignment or the grid acceleration rate (Methods). Figure 1 shows an example where two samples (Methods and Table 1) were deposited onto the grid by setting the second stream of sample droplets to initially overlap the first stream at the leading edge of the grid and then separate from it by 1–3 squares near the grid’s trailing edge toward the end of sample deposition. This allows comparison of the unmixed control and mixed experiments on the same grid. In the non-overlapping regions, we observed that apoferritin or 70S ribosomes were in high concentrations and well distributed in the vitreous ice of the individual.

Volumes on the order of 50 pl have been shown to mix completely within ~10 ms when brought together in midair just before colliding with a surface; thus, good mixing of the droplets on the nanowire surface is expected. Nevertheless, as a first proof of principle, we mixed two test samples, apoferritin and 70S ribosomes. In practice, one can control the extent of sample stream overlap by adjusting either the dispenser head alignment or the grid acceleration rate (Methods). Figure 1 shows an example where two samples (Methods and Table 1) were deposited onto the grid by setting the second stream of sample droplets to initially overlap the first stream at the leading edge of the grid and then separate from it by 1–3 squares near the grid’s trailing edge toward the end of sample deposition. This allows comparison of the unmixed control and mixed experiments on the same grid. In the non-overlapping regions, we observed that apoferritin or 70S ribosomes were in high concentrations and well distributed in the vitreous ice of the individual.

We have developed a spraying-mixing approach based on the Spotiton robot that uses a piezo dispensing tip to apply a stream of ~50-pl droplets onto a nanowire (‘self-wicking’) grid as it rapidly speeds past on its way to vitrification, resulting in a stripe of ice of fairly uniform thickness across each grid. The fast spot-to-plunge time can reduce the deleterious effects of the air–water interface and has been used to prepare grids for a wide variety of protein samples. By adding a second piezo dispensing tip to the device, we can deliver a second stream of droplets onto the first stream within 10 ms of it being deposited. The two sample volumes mix on the grid as the bulk volume is wicked away and spread out to a thin film by the capillary action of the nanowires. Currently, the dual-dispenser system can produce useful grids across a range of mixing time intervals from ~90–500 ms. Changes to the physical design of the machine and improving the wicking capacity of the nanowire grids should allow for the reduction of the lower limit to ~50 ms.

Here we describe the spraying-mixing method and demonstrate its efficacy and value for four biological systems where short-lived states are of high interest: (1) binding of ribosomal subunits; (2) binding of promoter DNA to RNA polymerase (RNAP); (3) binding of Ca2+ to a potassium channel followed by a conformational change; and (4) conformational rearrangements of dynamin lipid tubes driven by GTP hydrolysis. The biological systems reveal interesting changes in conformations, but we note that further work will be required to determine if the kinetics and thermodynamics are altered by adsorption to the air–water interface, an inevitable result of vitrification of thin films.

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device followed by a 140-ms delay in a reaction chamber. This difference in assembly states is not surprising as the rate of interactions is caused by the 17 N-terminal residues obstructing permeation in a ball-and-chain manner. Single-particle cryo-EM analyses of open channels and other intermediates, completing after the channel opens but before it inactivates, would reveal MthK gating ring structure (Extended Data Fig. 3b). In addition, ligand-binding domain revealed that it corresponded to the open structure obtained without Ca\(^{2+}\) tilting with respect to the ligand-binding domain (Fig. 2b). This tilt was an open-state signature and was different from the closed densities for Ca\(^{2+}\) within ~100 ms of Ca\(^{2+}\) activation, as the density for the transmembrane domains within the nanodiscs is weak, these results indicate that Ca\(^{2+}\) yields structures of closed and inactivated channels, respectively. The prediction was that freezing MthK within ~100 ms of Ca\(^{2+}\) application, after the channel opens but before it inactivates, would reveal structures of open channels and other intermediates, completing the gating cycle. Using time-resolved Spotiton to mix MthK and Ca\(^{2+}\) at ~150 ms, the structure had the transmembrane domain tilted with respect to the ligand-binding domain (Fig. 2b). This tilt was an open-state signature and was different from the closed structure obtained without Ca\(^{2+}\) (ref. 22; Fig. 2b). Analysis of the structure change to an activated state.

As another example of the use of Spotiton to capture biologically critical isomerizations, we investigated the initial steps of transcription between subunits is expected to be much slower via diffusion within droplets that mix on the grid rather than by the mixing that occurs by chaotic advection in the device described in the earlier study. We also note that the previous study used a higher concentration of 30S subunits, which was twice that of 50S subunits, whereas the data shown here used a 1:1 ratio of 30S to 50S subunits.

### Table 1 | Spotiton sample preparation conditions

| Experiment | Sample (concentration in dispenser) |
|------------|------------------------------------|
| Apoferritin + 70S mixed | Tip 1 Apoferritin (2.3 mg ml\(^{-1}\)) |
| 50S + 30S mixed | Tip 1 50S (1.4\(\mu\)M) |
| 50S or 30S only | Tip 1 50S or 30S (1.4\(\mu\)M) |
| MthK + Ca\(^{2+}\) mixed | Tip 1 MthK (12 mg ml\(^{-1}\)) and Fos8-F (3 mM) |
| MthK only | Tip 1 MthK (12 mg ml\(^{-1}\)) and Fos8-F (3 mM) |
| RNAP + DNA mixed | Tip 1 DNA (24\(\mu\)M) and beta-OG (0.35%) |
| RNAP only | Tip 2 RNAP (8 mg ml\(^{-1}\)) and beta-OG (0.35%) |
| Dynamin tubes + GTP mixed | Tip 1 Dynamin tubes |
| Dynamin only | Tip 2 GTP (2 mM and 4 mM) |

\(\beta\)-OG, n-octyl-\(\beta\)-D-glucoside; Fos8-F, (1H, 1H, 2H, 2H-perfluorooctyl)phosphocholine (Anatrace).

Fig. 1 | Apoferritin and 70S ribosomes were used as a proof of principle to illustrate mixing on the nanowire grids. a. Overview of the vitrified grid showing the sample streams merged at the leading edge (bottom) and separated at the trailing edge (top). Squares containing vitrified ice are indicated by a white outline; scale bar, 100 \(\mu\)m. b-d. Micrographs obtained from the indicated regions show apoferritin (b), 70S ribosomes (c) or a mix of both samples (d) and are representative of approximately 470 images each; scale bar, 100 nm.
initiation. Formation of the transcriptionally competent ‘open’ complex (RPo) is a dynamic, tightly regulated process requiring multiple steps. For *Escherichia coli* RNAP, mechanistic studies demonstrated that intermediates on the pathway of DNA opening interconvert on the timescale of subseconds to seconds. Although multiple intermediates in RPo formation were identified decades ago, their transient nature has prevented atomic resolution structural characterization as they interconvert in time. Here, we examined DNA opening by *E. coli* RNAP holoenzyme at the λPR promoter using time-resolved Spotiton. The kinetics of RPo formation at λPR have been extensively characterized, allowing predictions of intermediate populations as a function of time and solution conditions. Within the ~150-ms time of mixing and freezing, only the earliest intermediates were predicted to be present. The two-dimensional (2D) class averages from this experiment show DNA bound to RNAP alone or with promoter DNA. Future experiments that vary time, solution conditions and promoter sequence combined with three-dimensional (3D) classification strategies are anticipated to reveal the on-pathway nucleation and propagation of the transcription bubble.

Finally, we looked at the dynamics of dynamin at the ~150-ms timescale. During GTP hydrolysis, dynamin constricts and pinches off invaginating clathrin-coated vesicles. Previous results have shown that dynamin constricts the membrane within

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**Fig. 2 | Four examples of biological systems in which time-resolved cryo-EM provides answers.**

*a*, Left, micrograph from the mixed region of a Spotiton-prepared grid shows 30S and 50S ribosomal subunits and 70S complexes and is representative of 311 images (Table 2); scale bar, 100 nm; right, corresponding 2D classes show particles representing a population of 30S (yellow), 50S (blue), 50S-50S dimers (turquoise) and 70S (magenta). Approximately 20% of particles were reconstructed to a 70S complex at a resolution of 4.75 Å (Methods and Extended Data Fig. 2); scale bar, 20 nm. 

*b*, 3D volumes generated from MthK in the presence (top left) or absence (top right) of calcium showing clear differences in the overall conformation of the channel. The bottom row shows one of the three Ca²⁺ binding sites in MthK either occupied in the case of a mixing experiment (left) or vacant as with the MthK only control (right).

*c*, Representative class averages of RNAP alone (top) or mixed with promoter DNA (bottom) showing DNA clearly bound (blue arrowheads); scale bar, 10 nm.

*d*, Measured diameters (mean ± s.d.) of dynamin-decorated tubes without GTP (control, 43.85 ± 5.86, n = 48) and with GTP (2 mM, 38.80 ± 3.2, n = 48; 4 mM, 40.40 ± 4.23, n = 48); **P = 0.0014 and ****P = 0.0001, two-sided Student’s t-test.
seconds, and during this process the helical parameters transform from a one-start to a two-start helix\textsuperscript{26,27}. However, the rate and mechanism of how the dynamin polymer constricts and rearranges during GTP hydrolysis remain unknown. Using time-resolved Spottion to mix preformed dynamin tubes with GTP, we observed that, at ~150 ms, a high percentage of the dynamin-decorated tubes were constricted (that is, the lumen of the lipid bilayer was reduced) to 39 nm after mixing with 2 mM GTP compared to a constriction of 44 nm for untreated controls (Fig. 2d). After mixing with 4 mM GTP, the dynamin polymer became disordered, constricted and disassembled from the lipid bilayer (Fig. 2d and Extended Data Fig. 4). This work provides important clues to the initial steps that lead to dynamin-mediated membrane constriction and fission. We expect that further analysis incorporating a decreasing range of GTP concentrations will trap the reaction at the slowest step, allowing changes in the dynamin organization during early fission events to be observed.

These four biological cases represent a range of short-lived molecular states of high interest and demonstrate that samples can be successfully mixed on a grid and rapidly vitrified within ~100 ms to trap intermediates. This method uses very small quantities of material and is applicable to mixing together any two, or potentially more, samples to allow the capture of short-lived molecular states that appear between 50–500 ms after an interaction.

Online content
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References
1. Frank, J. Time-resolved cryo-electron microscopy: recent progress. J. Struct. Biol. 200, 303–306 (2017).
2. Berriman, J. & Unwin, N. Analysis of transient structures by cryo-microscopy combined with rapid mixing of spray droplets. Ultramicroscopy 56, 241–252 (1994).
3. White, H. D., Walker, M. L. & Trinick, J. A computer-controlled spraying-freezing apparatus for millisecond time-resolution electron cryomicroscopy. J. Struct. Biol. 121, 306–313 (1998).
4. Unwin, N. & Fujioyoshi, Y. Gating movement of acetylcholine receptor caught by plunge-freezing. J. Mol. Biol. 422, 617–634 (2012).
5. Subramaniam, S. et al. Protein conformational changes in the bacteriorhodopsin photocycle. J. Mol. Biol. 287, 145–161 (1999).
6. Lu, Z. et al. Gas-assisted annular microsprayer for sample preparation for time-resolved cryo-electron microscopy. J. Micromech. Microeng. 24, 115001 (2014).
7. Lu, Z. et al. Passive microfluidic device for sub millisecond mixing. Sens. Actuators B Chem. 144, 301–309 (2010).
8. Fu, Z. et al. Key intermediates in ribosome recycling visualized by time-resolved cryo-electron microscopy. Structure 24, 2092–2101 (2016).
9. Jain, T., Sheehan, P., Crum, J., Carragher, B. & Potter, C. S. Spottion: a prototype for an integrated inkjet dispense and vitrification system for cryo-TEM. J. Struct. Biol. 179, 68–75 (2012).
10. Dandy, V. P. et al. Spottion: new features and applications. J. Struct. Biol. 202, 161–168 (2019).
11. Wei, H. et al. Optimizing ‘self-wicking’ nanowire grids. J. Struct. Biol. 202, 170–174 (2018).
12. Scapin, G. et al. Structure of the insulin receptor–insulin complex by single-particle cryo-EM analysis. Nature 556, 122–125 (2018).
13. Zhang, Z. et al. Ensemble cryo-EM elucidates the mechanism of insulin capture and degradation by human insulin degrading enzyme. eLife 7, e35572 (2018).
14. Han, H. et al. Structure of Vps4 with circular peptides and implications for translocation of two polypeptide chains by AAA+ ATPases. eLife 8, e40791 (2019).
15. Liu, Y. et al. FACT caught in the act of manipulating the nucleosome. Nature 577, 426–431 (2020).
16. Noble, A. J. et al. Routine single-particle Cryo-EM sample and grid characterization by tomography. eLife 7, e42578 (2018).
17. Wu, J. L. Y., Tellkamp, F., Kuhajdoupur, M., Robertson, W. D. & Müller, R. J. Rapid mixing of colliding picoliter liquid droplets delivered through-space from piezoelectric-actuated pipettes characterized by time-resolved fluorescence monitoring. Rev. Sci. Instrum. 90, 055109 (2019).
18. Lu, Z. et al. Monolithic free-microfluidic mixing devices for time-resolution cryo-electron microscopy. J. Struct. Biol. 168, 388–395 (2009).
19. Chen, B. et al. Structural dynamics of ribosome subunit association studied by mixing-spraying time-resolved cryogenic electron microscopy. Structure 23, 1097–1105 (2015).
20. Zadek, B. & Nimigean, C. M. Calcium-dependent gating of MthK, a prokaryotic potassium channel. Nature 580, 288–293 (2020).
21. Ruff, E. F., Record, M. T. J. & Artisovitch, I. Initial events in bacterial transcription initiation. Biomolecules 5, 1035–1062 (2015).
22. Fan, C. et al. Ball-and-chain inactivation in a calcium-gated potassium channel. Nature 580, 288–293 (2020).
23. Mazumder, A. & Kapanidis, A. N. Recent advances in understanding σ7-dependent transcription initiation mechanisms. J. Mol. Biol. 431, 3947–3959 (2019).
24. Saecker, R. M., Record, M. T. J. & Dehaseth, P. L. Mechanism of bacterial transcription initiation: RNA polymerase-promoter binding, isomerization to initiation-competent open complexes, and initiation of RNA synthesis. J. Mol. Biol. 412, 754–771 (2011).
25. Sundborger, A. C. et al. A dynamin mutant defines a superconstricted prefission state. Cell Rep. 8, 734–742 (2014).
26. Woller, W. et al. Force-evoked structural transition in the dynamin polymer. Proc. Natl Acad. Sci. USA 108, 2628–2633 (2011).
27. Kong, L. et al. Cryo-EM of the dynamin polymer assembled on lipid membrane. Nature 560, 258–262 (2018).

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Methods
Spotiton instrument. The Spotiton system\textsuperscript{9,10} was upgraded with a second set of identical dispense-head components. A second piezo-driven electric tip was mounted next to the first head at 4.5 mm from the first tip (Extended Data Fig. 1b). The 4.5 mm pitch allowed the two tips to simultaneously aspirate samples from two adjacent holder tubes, which were also mounted at a 4.5 mm pitch. The second tip included a manual fine-adjustment screw to allow precise alignment between the two tips in the direction perpendicular to the plunge-axis motion.

The second piezoelectric tip was driven by an independent electronic drive (DE03 controller) that was added to the system. The plunge axis output is a series of electrical pulses while plunging (distance between pulses is a parameter set to 0.25 mm). The plunge-axis pulse output is tied to the trigger input of both DE03 controllers. Each DE03 controller can be set up to start firing its respective tip after a unique number of pulses (configurable by the user) relating to the position of the plunge axis. The fluidics of the second piezoelectric tip were attached to a second syringe pump that was added to the system. The syringe pumps allow precise independent sample aspiration and cleaning of the tips.

Time resolution. The motion path (136 mm) of a grid prepared by the Spotiton time-resolved system is characterized by three phases: acceleration, constant velocity and deceleration (Extended Data Fig. 1a). The durations of the acceleration and constant-velocity phases are variable and dependent on the rate of acceleration set by the user. The deceleration phase remains fixed and ends when the grid comes to a stop in the liquid ethane cup. The two dispensers spray a defined number of sample droplets whose first contact with the falling grid is separated by a period of 20–50 ms (~80–160 ms depending on the acceleration rate selected (Supplementary Table 4)). The first sample thus has a brief opportunity to be wicked away by the capillary action of the nanowires before contact by the second sample (Extended Data Fig. 1b). The mixing time of the two samples before vitrification ranges from 130–160 ms but can be reduced to ~90 ms by moving the dispensers to a ‘low-fire’ position ~4 cm closer to the ethane bath (Condition 4 in Supplementary Tables 1 and 2). A redesign instrument would, in principle, be capable of even shorter mixing times; for example, the commercial version of the Spotiton chameleon (SPT Labtech) is capable of spot-to-plunge times of ~50 ms.

Time-resolved Spotiton operation. A typical protocol for operating the time-resolved Spotiton system proceeds as follows. On start-up, the system is initialized, and the two three-axis robots used to position the grid-holding tweezers and the dispensing heads are homed. Next, the fluid lines carrying distilled water from an external reservoir to the dispensing tips are flushed several times to remove air bubbles and any residual menstralin used to clean the tips after the previous session. Both tips are then fired in view of an inspection camera to confirm successful dispensing and the formation of discrete droplets. Next, a standard (not nanowire) test grid is loaded into the tweezers, lowered into position between the upper camera and the upper dispenser tip and both are observed for alignment in the live viewer in the main software window. Aided by an integrated image-recognition algorithm, the operator positions the tip along the vertical midline and at the leading (lower) edge where the first-dispersed droplets will contact the grid. The second piezo device is locked into position directly beneath the first, but its tip is steerable to allow the second sample to be dispensed either completely overlapping the first sample stripe or in a discrete, non-contacting parallel stripe. To verify the system operation, each tip is fired separately on the test grid, and video captures from the upper camera are examined to confirm deposition of a liquid stripe (analogous to the humidity of the chamber is increased to 80–85% by activating the in-chamber humidity controller) that was added to the system. The plasma pumps allow precise independent sample aspiration and cleaning of the tips.

Sample preparation. A series of experiments was performed to first test and verify mixing of protein samples on the grids and then to demonstrate the value of this approach for a variety of biological systems of interest. In general, vitrified grids of mixed samples were prepared as follows: nanowire grids were freshly plasma cleaned and transferred into the enclosed Spotiton chamber (room temperature, 0–85% humidity) no more than 30 s before vitrification. Approximately 5 μl of each sample was loaded into the two sample-holder cups with the concentrations reported in Table 1. For control experiments, the second sample was replaced by the carrying buffer as noted. The calculated spot-to-plunge time for all of the grids is 151 ms. Below, we briefly describe further details of sample and grid preparation for each of these experiments.

Apoferotinin and 70S ribosomes. Apoferotinin was purchased from Sigma-Aldrich (400kDa, A3660, 2.3 mg ml\textsuperscript{−1}). Protein solution stored in 50% glycerol was exchanged into a cryo-compatible buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl) using Amicon Ultra (100 kDa cutoff membranes). We used 70S ribosomes from New England BioLabs (2MDa). Protein solution was stored in 20 mM HEPES-KOH (pH 7.6), 10 mM Mg(CH\textsubscript{3}COO), 30 mM KCl and 7 mM beta-mercaptoethanol after diluting the sample to 1 mg ml\textsuperscript{−1} from 33.3 mg ml\textsuperscript{−1}.

30S and 50S ribosomal subunits. Next, 70S ribosomes were prepared as described in Johansson et al.\textsuperscript{11}. For subunit purification, 70S ribosomes were exchanged into dissociation buffer (20 mM MES-KOH (pH 6), 600 mM KCl, 8 mM Mg(CH\textsubscript{3}COO), 1 mg ml\textsuperscript{−1} heparin, 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM benzamidine and 2 mM dithiothreitol (DTT)) before loading onto a sucrose gradient in the same buffer and centrifuged for 19 h at 28,300 r.p.m. in the Ti25 rotor. The 50S and 30S subunits were exchanged separately into reassociation buffer (10 mM MES-KOH (pH 6), 10 mM NH\textsubscript{4}CH\textsubscript{3}COO, 40 mM CH\textsubscript{3}COOK, 8 mM Mg(CH\textsubscript{3}COO), and 2 mM DTT), concentrated to 6 μM and stored at ~80 °C after being flash frozen in liquid nitrogen.

RNP and promoter donor. Core RNP (subunit composition αββ\textsubscript{0}σ) was expressed and purified as described\textsuperscript{12} with the following modifications: (1) a plasmid encoding His\textsubscript{6}-SUMO-\textsuperscript{e} was used instead of His\textsubscript{10}-SUMO-\textsuperscript{e}; (2) cells were grown at 30 °C in the presence of 50 μg ml\textsuperscript{−1} kanamycin until optical density (OD) reached 0.4, then temperature was lowered to 16 °C, and (3) when the cells reached an OD\textsubscript{600}=0.1, a lysozyme (1 mg ml\textsuperscript{−1}) was added to the culture to aid in continued for an additional 15 h. After harvest by centrifugation and resuspension in lysis buffer\textsuperscript{13}, cells were flash frozen in liquid nitrogen and stored overnight at ~80 °C. Cells were thawed halfway at 22 °C, thawed completely on ice and then lysed in a French press. After lysis, the series of columns and buffers used to prepare RNAP to a more purified state were added. For promoter DNA, a duplex IP promoter fragment (~85 to +420) was used (Trilink Biotechnologies); top (non-template) strand: 5′-GCC GAG ATC GAG TCC TCT ACA GAT GGA TAA ATA CTA AAC ACC GTC CGT GGT GAC TAT TTT ACC TCT GGC GGT GAT AAT GGT TGC ATG TAC TAA GGA GGT TGT G A 3′; bottom (template) strand: 5′-CTA ACC TCG TTA GEA CAT GAC ATT ATC ACC GCC AGA GGT AAA ATC ATA AAC AGC CAG GGT GAT GGA TAT GTA TCC AAT CTC AAC GGA TCC CTC GAT ACC GAG CTC GAT AAT CTC GCC AAG CAG CCC GGT GTT GC TAT G 3′. RNAP holozyme was assembled by mixing core enzyme with a 3.3-fold molar excess of σ'\textsuperscript{14}, incubating for 20 min at 37 °C and buffer exchanging into gel filtration (GF) buffer (40 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM MgCl\textsubscript{2} and 10 mM DTT) using centrifugal filtration (Amicon Ultra 0.5 ml with 30k cutoff) at 4 °C. Excess σ'\textsuperscript{14} was separated from core RNAP on a Superose 6 increase 10/300 GL column (GE Healthcare) equilibrated in GF buffer. The eluted fractions of RNAP were concentrated to 16 mg ml\textsuperscript{−1} (centrifugal filtration), aliquoted, flash frozen in liquid nitrogen and stored at ~80 °C. The non-template and template strands of IP promoter DNA were dissolved in annealing buffer (10 mM Tris-HCl (pH 8), 50 mM KCl and 0.1 mM EDTA), mixed in equimolar amounts and incubated in a 95 °C heat block for 10 min. The samples were then slow cooled in the heat block to room temperature. Annealed DNA was stored at ~80 °C. RNAP and DNA aliquots were thawed on ice and diluted to the concentrations reported in Table 1 with GF buffer. Thereafter, n-octyl–β-D-glucopyranoside (Anatrace) was added to a final concentration of 0.35% just before spraying at room temperature.

Cat\textsuperscript{−}/gated MthK channel. MthK was purified and reconstituted into nanodiscs composed of POPE:POPG (3:1) lipids, following the procedure described in detail previously\textsuperscript{15}.
Dynamin tubes and GTP. For liposome formation and dynamin purification, 1,2-dioleoyl-sn-glycerol-3-phospho-1-serine (100 μg of ml−1 DOPS, Avanti) was dried and resuspended in 250μl HCB150 (50 mM HEPES, 150 mM KCl, 2 mM EGTA, 1 mM MgCl2, and 1 mM TCEP (pH 7.5)). Unilamellar liposomes were obtained by extruding the mixture 21 times through a 0.4-μm-pore polycarbonate membrane (Avanti). Recombinant AP-3β-dynamin 1 was purified from S9 insect cells. Briefly, recombinant baculovirus containing the sequence of AP-3β-dynamin 1 with an N-terminal His-tag was generated by following the Bac-to-Bac Baculovirus Expression System (Thermo Fisher Scientific). The suspension cultures of S9 were maintained in SF-900 III serum-free medium (SEM, Thermo Fisher Scientific) and inoculated with recombinant baculovirus at a cell density of 1.6 × 10^6 with a 1:100 volume of virus to final volume of medium. The cells were grown for 72 h at 27 °C and pelleted by centrifugation at 100 x g for 30 min at 4 °C. The pellet was resuspended in 3× of modified HSBI50 (50 mM HEPES, 150 mM KCl, 5 mM beta-mercaptoethanol and 10 mM imidazole (pH 8.0)) with a protease inhibitor cocktail (Millipore Sigma). The cells were then lysed by sonication (8 min in total; pulse on/off: 5 s/15 s) followed by high-speed centrifugation (20,000 g) for 15 min. The supernatant was collected, passed through nickel-nitriotropic acid (Ni-NTA) beads and the protein was eluted with 150 mM imidazole in modified HSBI50. The protein solution was dialyzed in HSBI50 overnight, and the purity was checked using SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie staining. Dynamin–decorated tubes were generated by incubating 3 μl of DOPS liposomes with 40 μl of protein (0.8 mg ml−1) in 10 mM Tris buffer, 10 mM KCl and 1 mM MgCl2 (pH 7.4) for 2 h.

Imaging and analysis. Typically, data were acquired using Leginon MS30, and CryoSPARC non-uniform refinement to generate a structure with an overall resolution was 4.1 Å for Mthk with Ca2+. Typically, data were acquired using Leginon MS30, and CryoSPARC non-uniform refinement to generate a structure with an overall resolution was 4.1 Å for Mthk with Ca2+. Briefly, 2,158,345 particles were automatically picked and used for two rounds of 2D classification using MotionCor2 (ref. 31), and contrast transfer function (CTF) was estimated using CTFIND4 (ref. 32). Particle picking was performed with Gautomatch (https://www.mrc-lbm.cam.ac.uk/kxhang/) and particles were extracted in Relion in 5× binning followed by one round of 2D classification using Relion11 to remove false particles. After the first round of 2D classification, classes clearly representing S05 dimers and S05 and S05 particles (Fig. 2a) were excluded from further steps of image analysis. After one round of 3D classification, only recognizable 70S particles were selected and re-extracted to a pixel size of 2.2 Å for final refinement. A total of 26,402 particles were used for homogeneous 3D refinement in Relion resulting in a 4.8-Å map of the 70S ribosome (Extended Data Fig. 2a,b). For the control experiments, a procedure similar to that described above was used to obtain a total of 12,505 and 3,762 individual S05 and S05 particles, respectively, and particles were analyzed by 2D classification in Relion (Extended Data Fig. 2c,d).

MTHK with and without Ca2+. Typically, a small set of particles was manually picked, and 2D class averages were calculated using the CL2D algorithm11 inside the Appion image processing pipeline11. A subset of these classes was used as a template to pick particles for the entire set of micrographs using FindEM36. For Mthk without Ca2+, 956,882 particles underwent several rounds of 2D classification and ab initio reconstruction using CryoSPARC33. From these, 428,917 particles were used for final 3D classification, and the best class was selected and used for CryoSPARC uniform refinement to generate a structure with an overall resolution of 4.2 Å (Fig. 3b). For Mthk with Ca2+, we used similar procedures to those described above. Briefly, 2,158,345 particles were automatically picked and used for two rounds of 2D classification in Relion. These from 849,864 good particles were selected and used for 3D classification in Relion. The open-state class with a highly tilted regulator of potassium conductance (RCK) domain was selected and used for CryoSPARC non-uniform refinement to generate a structure with an overall resolution of 6.3 Å (Fig. 2b). For the focused refinement of the RCK domain, signal-subtracted particles of both samples were generated with a mask to only include the RCK domain. These particles were used for refinement in Relion by applying C2 symmetry, and the overall resolution was 4.1 Å for Mthk with Ca2+ and 3.5 Å for Mthk without Ca2+ (Extended Data Fig. 3b).

RNAP with and without DNA. Particle picking was performed with Gautomatch, and particles were extracted in Relion, followed by one round of 2D classification to remove false picks using CryoSPARC33. Approximately 167,212 particles of RNAP with DNA and 52,747 particles of RNAP alone were selected for another round of 2D classification, and the 2D classes with high-resolution features were selected (Fig. 2c).

Notes: For all experiments, the following references were used: 11. Chen, J. et al. EMStructures: a comprehensive database of high-resolution structures from single molecules. eLife 7, e11633, 2018. 12. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
Extended Data Fig. 1 | Specifications of time-resolved Spotiton operation. a, Diagrammatic overview of the distances (fixed) and elapsed times (variable) relevant to spraying and mixing two samples on a moving grid. Simultaneous dispensing of both samples is triggered after the grid plunge begins. Representative images from the upper and lower cameras are shown directly below the illustrations of each. Sample 1 and sample 2 are indicated in blue and yellow, respectively. b, Magnified view of (green-dashed) boxed area in (a) showing grid and dispensing at specific time-points with corresponding high-speed video captures of the tips and grid below. Elapsed times shown on each image reflect estimates from a video of a grid plunged under Condition 2 (Supplementary Table 1). Objects in (a) and (b) are not drawn to scale. Supplementary Tables 1–4 list values for the following parameters of a grid plunged as depicted in (a) and (b): $a_{\text{acc}}$, acceleration rate; $a_{\text{decc}}$, deceleration rate; $v_{\text{max}}$, maximum velocity; $t_s$, plunge start point; $t_{\text{disp-1}}$, grid leading edge reaches first dispenser; $t_{\text{samp-1}}$, sample 1 fully applied to grid; $t_{\text{disp-2}}$, grid leading edge reaches second dispenser; $t_{\text{samp-2}}$, samples 1 and 2 fully applied to grid; $t_{\text{UC}}$, grid reaches upper camera, $t_{\text{LC}}$, grid reaches lower camera; $t_e$, grid plunges into ethane. ‘Spot-to-plunge’ and ‘mix-to-plunge’ in (a) reflect the elapsed times from $t_{\text{disp-1}}$ or $t_{\text{samp-2}}$ to $t_e$, respectively.
Extended Data Fig. 2 | Mixing 30 S and 50 S ribosomal subunits to form 70 S complexes. a, ~20% of particles present were reconstructed to 70 S complex at a resolution of 4.75 Å as indicated by FSC0.5 (b). c, 2D classes of 50 S ribosomal subunit obtained from the control experiment; 2D class of 50S-50S dimer is shown in red. d, 2D classes of the 30 S ribosomal subunit obtained from the control experiment. Both control experiments show no evidence of 70 S ribosomes as observed in the mixed experiment. Scale bars, 20 nm.
Extended Data Fig. 3 | Cryo-EM maps of MthK RCK domain with and without Ca\(^{2+}\): a. The two additional Ca\(^{2+}\) binding sites of MthK either vacant from a control experiment (top row) or occupied after mixing with calcium (bottom row). b. 3D volumes of MthK RCK domains without (top row) and with (bottom row) Ca\(^{2+}\) bound.
Extended Data Fig. 4 | Mixing of GTP with dynamin-decorated lipid tubes results in constriction. Representative cryo-electron micrographs of control dynamin-decorated tubes without GTP (a), with 2 mM GTP (b) and 4 mM GTP (c). Scale bars, 50 nm.
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Sample size: These items are not relevant for this study as it deals with technical method development and protein structural data.

Data exclusions: Data were not excluded from the analyses.

Replication: The chief aim of this study was to demonstrate mixing of two samples at the sub-second timescale using the Spotiton system, rather than to conclusively illustrate any novel, biological phenomenon. As such, each of the five experiments conducted (1. Proof of principle, 2. 70S ribosome assembly, 3. RNAP binding DNA, 4. MthK conformational change, 5. Dynamin tube constriction) are independent ‘replicates’ that together conclusively support the chief aim of the paper, as stated above.

Randomization: As this paper dealt solely with technical method development, randomization does not apply.

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