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Time-resolved cryo-electron microscopy: Recent progress

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SUMMARY

Time-resolved cryo-electron microscopy (cryo-EM) combines the known advantages of single-particle cryo-EM in visualizing molecular structure with the ability to dissect the time progress of a reaction between molecules in vitro. Here some of the recent progress of this methodology and its first biological applications are outlined.

1. Quasi-stability of states in biology

Biology involves interactions of molecules in the cell, in a thermal environment compatible with physiological conditions. The molecules themselves contain mobile components that facilitate chemical reactions and the transport of moieties required in these processes. Through evolution, structures of molecules and their mobile components are designed such that energies of functionally relevant states are separated by low energy barriers. These are in a range that is accessible in the thermal environment. Where necessary, energy barriers are lowered by the binding of protein factors acting as enzymes. Building principles for interacting surfaces are governed by the need to avoid energy traps that would lead to locking the machine in one of its states (Frauenfelder, 2010). For instance, ribosomal subunits, once assembled into functioning ribosomes around a new genetic message, must be able to rotate against each other over almost ten degrees without losing connection during the process of translocation of mRNA and tRNAs (see Frank and Gonzalez, 2010). On the other hand, for them to rotate and interface in ever-changing binding constellations, all intermediate states must have shallow energy minima without major hurdles in between. Biology, in short, is based on, or sustained by, quasi-stability of structural assembly, and the ease with which different, closely neighboring functional states can interconvert.

As a consequence of this design, the functional states of a molecular machine possess different (temperature- and pH-dependent) lifetimes, in a large range of times. Importantly, many interesting ones fall in a range (0 to several thousand milliseconds) that is impossible or quite difficult to access by standard cryo-electron microscopy; i.e., the slow method of grid preparation whereby the sample, once mixed, is pipetted onto the grid and the grid is first blotted, then plunge-frozen. On the other hand, as many recent studies show, single-particle cryo-EM offers a unique way of inventorying and visualizing multiple coexisting states, and one wishes to harness it for application in studies of fast processes, as well.

An example for an interesting short-lived state is provided with a reaction that causes the post-termination ribosome to split into its subunits, catalyzed by successive binding of ribosome recycling factor RRF and EF-G·GTP to the post-termination ribosome (PostTC), and subsequent GTP hydrolysis on EF-G (Zavialov et al., 2005). During this reaction a short-lived intermediate exists in which both RRF and EF-G in the GDP state are bound to the ribosome, but splitting has not yet taken place. Kinetic simulations show that this state only lasts for approximately 200 ms (Fig. 2). Practically, this means that within a second, all ribosomes are split into subunits, and the most interesting state, in terms of providing information about the splitting and recycling mechanism, would be lost in the lengthy procedure of standard cryo-EM.

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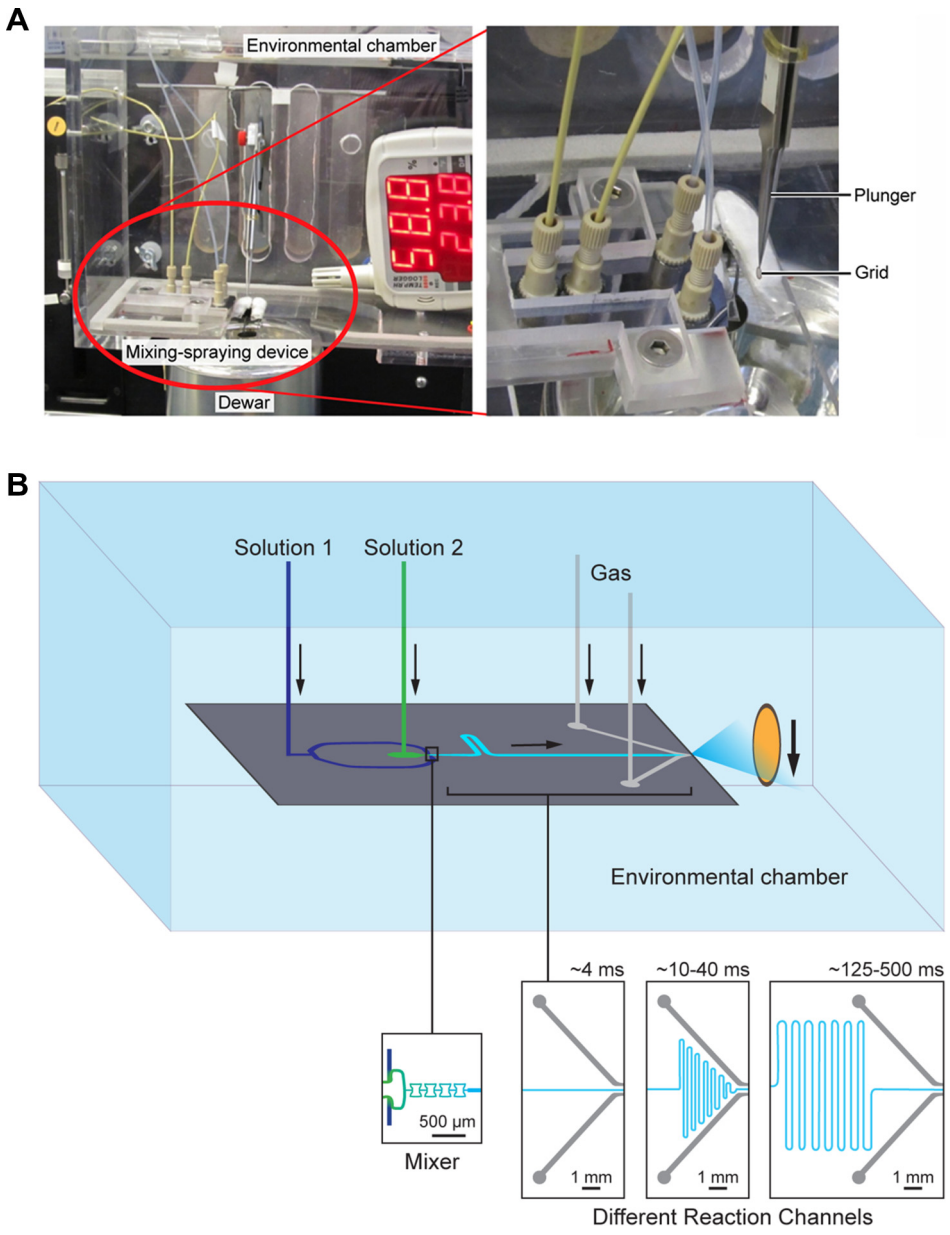

Fig. 1. Apparatus for time-resolved cryo-EM. (A) Close-up of the apparatus, and further close-up on the mounted chip with four inlets. The two inlets on the left conduct the two reactants into the chip; the two on the right both carry nitrogen gas under pressure, for operating the sprayer. (B) Schematic diagram of the microfluidic chip (Reproduced with permission from Chen and Frank, 2016).

2. Time-resolved cryo-EM

Time-resolved (TR) cryo-EM addresses the need for obtaining information about systems that are not in equilibrium but change over time, until equilibrium is reached. If this is a slow process, e.g., in the range of minutes, then all we have to do is start the reaction and then take samples in some intervals for visualization of the molecules with negative-staining EM (Mulder et al., 2010) or standard cryo-EM (Fischer et al., 2010). However, if the process is fast compared to the time required for pipetting and blotting (several seconds), then a special apparatus is needed which uses a microfluidic chip for mixing and reacting two reactants, and rapid deposition of the reaction product on the grid (Lu et al., 2009; Shaikh et al., 2014; Chen et al., 2015; Chen and Frank, 2016; Fu et al., 2016). We refer to this method as "mixing/spraying," in contradistinction to an earlier method of "spraying/mixing" developed by Berriman and Unwin (1994), in which one reactant is sprayed on a grid already covered with another reactant, and the mixing takes place on the grid itself. This earlier method, although perfectly adapted for the process is was designed to study – the response of the acetylcholine receptor to its substrate – is less suitable for mixing of large molecules as it offers little control over the mixing on the grid.

The mixing/spraying method of Lu et al. (2009) currently covers reaction times in the range of 10–1000 ms, but it may be possible in future designs to extend this range toward shorter times. Ultimately, the shortest time is defined by the time required to cool the sample down to liquid nitrogen temperature (~1 ms), but other "dead" times must be added: the time for mixing (<1 ms), and the geometry-dependent minimum times for spraying (<2 ms) and plunging (10–18 ms) of the grid. Here the larger figure for plunging results from the addition of a climate chamber (Chen et al., 2015).

The apparatus (Fig. 1) is built around a computer-operated syringe pumping and freeze-plunging device developed by White and

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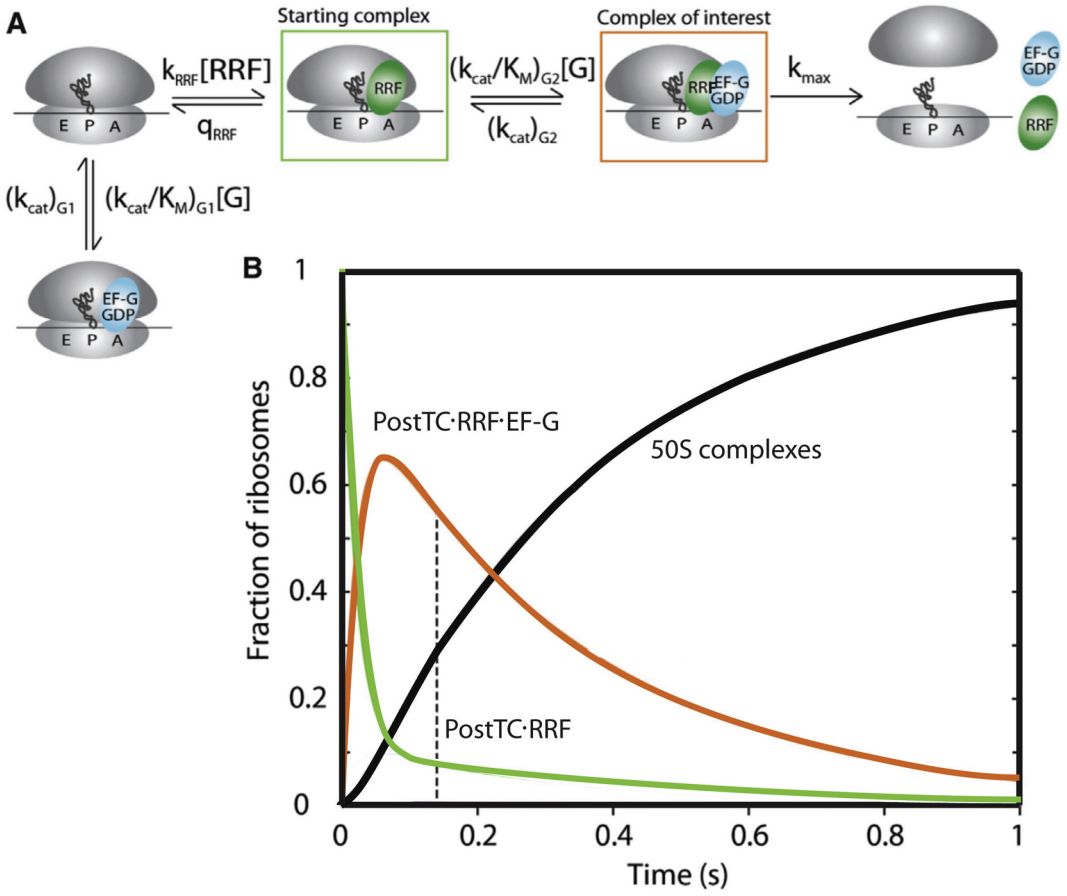

Fig. 2. The process of ribosome recycling. (A) Flow diagram depicting the reactions following the mixing of the starting complex PostTC·RRF with EF-G in the presence of GTP. (The branch on the left represents a non-productive path in which RRF falls off and is replaced by EF-G.) (B) Graph of kinetic measurements. The fraction of interesting short-lived intermediate PostTC·RRF·EF-G reaches a peak at ~70 ms and falls off within a second, bringing it out of reach of standard blotting/plunge-freezing cryo-EM. The time point caught by the microfluidic chip is indicated with a dashed line. The black curve depicts the rise in the fraction of separate 50S and 30S subunits (Adapted from Fu et al., 2016).

coworkers (White et al., 2003). It contains, within an environmental chamber (Chen et al., 2015), the microfluidic chip (currently silicon-based, as designed by Lu et al., 2009) with two inlets, a mixing chamber, a reaction channel of variable lengths, and a microsprayer which deposits the product on the EM grid as the latter passes through the spray cone on its way to the cryogen. In collaboration with Columbia University's Engineering Department, the chip is now being redesigned so it can be made from polydimethylsiloxane (PDMS), a much faster and cheaper process. The first stage of redesign has resulted in a self-standing PDMS-based microsprayer, which has now been tested in its ability to deposit droplets giving suitable ice thickness (Feng et al., 2017). Even as a self-standing sprayer, it might present a promising alternative to the pipetting/blotting method since it offers, in a certain range, control over ice thickness, by varying the ratio between liquid flow rate and driving gas pressure.

To return to the example of the ribosome recycling process cited above, a time-resolved experiment is started by mixing PostTC·RRF with EF-G·GTP. At different time points, four distinct subpopulations (namely PostTC·RRF, PostTC·RRF·EF-G·GTP, 50S subunit, and 30S subunit) are expected to occur with different ratios, with 70S·RRF·EF-G·GTP making its transient appearance within a time window from approximately 50–250 ms (Fig. 2), and eventually only 50S and 30S subunits will be left.

An article describing the sample preparation, data collection and outcome of this experiment has recently been published (Fu et al., 2016), and here it will suffice to give a brief summary reflecting on the gain in biological knowledge. A total of 84,000 particle images were collected from droplet areas for different time points. Sorting of these images with RELION, a program using a maximum-likelihood method to split the images into classes (Scheres, 2012), indeed reveals the presence of altogether seven distinct structures, which match the complexes expected (PostTC·RRF, PostTC·RRF·EF-G) but also show variants of the 30S and 50S subunits still bound with ligands being recycled (30S·tRNA, 30S·IF3; 50S·RRF·EF-G, 50S·RRF·EF-G·tRNA) (Fig. 3). Only complexes 2 and 7 have been observed before; all others are short-lived and hence inaccessible for standard cryo-EM or any other means of visualization.

The various complexes with their bound ligands were identified by segmentation and comparison with known structures of 70S, 30S, 50S, tRNA, RRF and EF-G. The most interesting result by far is the PostTC·RRF·EF-G complex (complex 1 in Fig. 3). It shows how EF-G binding and GTP hydrolysis causes intersubunit rotation and forces domain II of RRF to pivot toward the intersubunit bridge B2b, the bridge that is crucial for the stability of the 30S–50S association in the ribosome. Thus, we see the action that leads to the separation of the two subunits in the recycling process for the first time.

PostTC·$RRF_{control}$ (Fig. 3, complex 2) and NR-PostTC·RRF_{140} (Fig. 3, complex 3) represent two versions of the same complex, one showing intersubunit rotation, the other one without it. The rotated version dominates in the control. At 140 ms, by contrast, only the non-rotated version is seen, probably since the rotated

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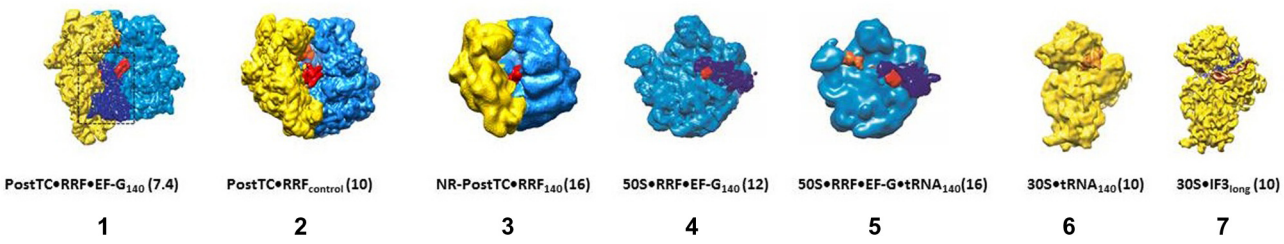

Fig. 3. Inventory of complexes found at 140 ms (complexes 1, 3–6), spray control (complex 2), and long-exposure control (complex 7). Ligands segmented out: EF-G (dark blue), RRF (red), E-site tRNA (orange), IF3 (brown). Resolution is stated in angstrom. The spray control sample was obtained by passing PostTC·RRF through one channel and buffer through the other. Long-exposure control was obtained by long-time (minutes) reaction and standard cryo-EM after passing the sample through one channel of the time-resolved apparatus, and buffer through the other (Based on Fu et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

version is capable of binding EF-G and going on pathway toward splitting, while the non-rotated form is apparently off pathway and therefore stuck.

The 50S subunit, one of the products of recycling, is bound with RRF and EF-G (in one form additionally with tRNA). It is likely that these two complexes are formed by the 50S subunit through re-binding of RRF and EF-G. The 30S subunit, the other product of recycling, exhibits a bound tRNA (and mRNA, not shown) at 140 ms but after a longer time span, the tRNA is replaced by IF3. This observation is relevant in the long-drawn controversy on the fate of tRNA and the role of IF3 in recycling (see discussion in Fu et al., 2016).

3. Conclusions

It is important to clarify what time-resolved cryo-EM of the kind described here can and cannot do. The ensemble of molecules imaged at any given time point will represent a sample of all co-existing states, and with the help of classification programs a few major, well-populated states can be identified. Thus what time-resolved cryo-EM can accomplish is to show how the molecule population redistributes into different states as time progresses. However, it cannot resolve domain motions (along trajectories of "microstates") as these occur on much shorter time scales.

I hope this brief account has demonstrated the potential of time-resolved cryo-EM in visualizing short-lived states that are important for the understanding of life processes but unobservable with standard cryo-EM. Through further investment in microfluidic engineering we might see an expansion of the time scale covered, in the direction below 1 ms, the development of versatile, tunable microfluidic chips, and reduction of minimum volume required in the experiment.

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