MICROSCOPY

*Microscopy*, 2016, 3–8
doi: 10.1093/jmicro/dfv358
Advance Access Publication Date: 12 November 2015

Review

# Generalized single-particle cryo-EM – a historical perspective

**Joachim Frank**[1,2,*]

[1]HHMI, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY, USA, and [2]Department of Biological Sciences, Columbia University, New York, NY, USA

*To whom correspondence should be addressed. E-mail: jf2192@cumc.columbia.edu

Received 7 October 2015; Accepted 15 October 2015

## Abstract

This is a brief account of the earlier history of single-particle cryo-EM of biological molecules lacking internal symmetry, which goes back to the mid-seventies. The emphasis of this review is on the mathematical concepts and computational approaches. It is written as the field experiences a turning point in the wake of the introduction of digital cameras capable of single electron counting, and near-atomic resolution can be reached even for smaller molecules.

**Key words:** 3D reconstruction, image processing, ribosome, molecular structure

The spectacular, fast-paced advances in single-particle cryogenic electron microscopy (cryo-EM) in the past 3 years, following the introduction of novel direct detection device (DDD) cameras with superior signal-to-noise ratio and resolution [1,2], are currently the subject of many commentaries and news and views articles conveying the general excitement of the structural biology community and the scientific community at large. As someone who has been in the field since the early days, I would like to contribute by a recollection of how the field developed.

Single-particle EM, as a novel approach to structural biology, required a heretical concept going against the grain of wisdom which held that quantitative structure determination by 3D reconstruction from EM projections [3] is not feasible unless molecules are arranged in crystalline order. These ordered structures include those with helical symmetry, as in DeRosier and Klug's reconstruction of a bacteriophage tail [3], arranged in a two-dimensional crystal [4,5], or with high symmetry as in viruses [6]. Viruses, although classifiable as single particles, contain structural information in a highly redundant form. For instance, the projection of a virus with icosahedral symmetry contains 60 projections of its asymmetric unit. Not only is the spatial arrangement of these units fixed and recoverable from the Fourier transform, but the signal is also retrieved with an instant bonus of nearly 8-fold reduction in the power of noise. Crowther and coworkers, in developing the common lines approach for recovering the structure of an icosahedral virus from its projection, did state that 'there is in principle no reason why the method should not be extended to systems with lower symmetry' [6], but it was not apparent how this could be practically accomplished for objects lacking symmetry altogether.

At the time, the idea of single-particle averaging of entirely asymmetric molecules [7] therefore created some excitement: *If such*, [i.e. asymmetric single-particle] *methods were to be perfected, then, in the words of one scientist, the sky would be the limit* [8]. Thus, Robinson's Research News article started with an appreciation of Unwin and Henderson's seminal work, the 3D reconstruction of bacteriorhodopsin

[9] and ended with curiosity and anticipation of a technique, then in its infancy, that would forgo the need for crystalline order or internal symmetry altogether.

An important early feasibility consideration was triggered by the question of how much radiation would be required to allow images of asymmetric molecules to be aligned by cross-correlation [10,11] as a prerequisite to averaging. The dose requirement was not at all clear from the outset but was of critical importance in assessing the viability of the entire approach. This study led to a quantitative expression linking particle size, contrast, critical dose (i.e. the maximum dose allowed, given what was known about radiation sensitivity of proteins from Robert Glaeser's studies [12]) and resolution obtainable. A demonstration of 2D averaging applying the newly minted SPIDER software on images of GroEL and 40S ribosomal subunits ensued [13,14]. SPIDER [15] was an early image processing package that, through its modular design and scripting language, made it possible to design complex workflows of single-particle data analysis from a menu of basic commands. Its dissemination over the years contributed to the popularization of the single-particle approach to molecular structure research.

Altogether, the realization of the single-particle approach with frozen-hydrated samples (hence, single-particle *cryo*-EM) required the convergence of three fundamental technical developments, which may be grouped roughly into specimen preparation, electron microscopy and mathematical/computational approaches. Each of these developments has its own history going back more than a few years: the art of specimen preparation was revolutionized by the frozen-hydrated sample preparation technique, ensuring that molecules be rapidly frozen and suspended in a thin layer of ice. This technique was pioneered by Dubochet [16], though drawing from earlier experiments with frozen-hydrated samples by Taylor and Glaeser [17].

On the instrument side, there were efforts to understand the mechanism of image formation, including the contrast transfer theory [4,18–21], and those, hand-in-hand, aimed to improve the performance of the transmission microscope and tailored to the needs of low-dose imaging of frozen-hydrated specimens. The latter required development of cryo-stages that ensure the maintenance of low temperature for the sample throughout the imaging. Specimen stages cooled with both liquid nitrogen and liquid helium were developed. (Although cooling all the way to 4°K turned out to be detrimental for single-particle techniques, because of an unexpected change in the physical properties of ice below 30°K under electron bombardment, the development of liquid helium stages [22] had a significant impetus on modern instrument design.) Another driving force was the implementation of protocols for low-dose electron microscopy, going back to the ground-breaking protocols of Unwin and Henderson for visualizing glucose-embedded 2D crystals of bacteriorhodopsin [5] and on early radiation damage studies by Glaeser [12]. Finally, the high brightness of field emission gun cathodes coming on the market in the 1990s ensured high spatial coherence, overcoming the damping of the object's Fourier transform due to partial spatial coherence as expressed by the envelope function [18].

While these developments in experimental techniques benefited cryo-EM as a whole, regardless of the type and symmetry of the specimen, the challenges posed in the analysis of projections of any free-standing, molecules negatively stained or suspended in ice, including molecules lacking any internal symmetry – the tenet of single-particle methods – required a radical rethinking and a departure from Fourier-based crystallographic approaches. These challenges are related to the fact that for asymmetric molecules, signal and noise are thoroughly intermixed in reciprocal space and not partially separated as for molecules in ordered arrangements. Evidently the key to separation of signal from noise is averaging, but as a prerequisite molecule projections need to be first aligned. Alignment of EM images using cross-correlation as a tool had been introduced early on [23].

However, any attempt to average molecule projections visible in an electron micrograph faced an obvious obstacle: for a molecule presenting multiple views, the selection of projections in any single view from the micrographs had to be done subjectively, by eye. This obstacle was overcome by the introduction of a then radically new tool, the representation of each molecule projection as a multidimensional vector [24] (Marin van Heel, a student of Ernst van Bruggen in Groningen, developed an interest in single-particle methods spelled out in my earlier publications and sought to work with me on the problem posed by heterogeneity during a series of visits in Albany). When a set of projections is aligned – by virtue of correlation methods developed earlier on, then their vectors all share the same multidimensional space, and clusters in that space reflect subpopulations of molecules presenting similar views, or, alternatively, molecules of different conformations and/or compositions. Thus multivariate statistical analysis and classification of aligned images [24] opened up the horizon for quantitative analysis of single asymmetric particles. The circumstances of this breakthrough – as the result of a fortuitous encounter with a laboratory scientist, Jean Pierre Bretaudiere, who used Correspondence Analysis to look for clustering of blood sample data among patients – have been recounted elsewhere [25]. Early examples for first applications of this technique were the sorting of images of 40S ribosomal subunits purified from HeLa cells [26] and of *Lumbricus* hemocyanin molecules [27].

Moving on to 3D reconstruction, most importantly, the spatial relationships among extremely noisy projections

must be established before they can be combined into a meaningful three-dimensional density map. Two *ab initio* solutions to this problem, posed initially by any unknown asymmetric molecule, were developed simultaneously: the random-conical reconstruction by my group [28,29] and the common-lines approach by other groups [30,31]. This latter approach was in fact an extension of the common lines approach developed earlier by Crowther *et al.* [6] for viruses with high symmetry, made possible by the improved signal-to-noise ratio afforded by 2D classification methods of van Heel and Frank [24]. The random-conical method, in contrast, employs a single tilt and makes use of preferred orientations of a molecule on the grid to establish an unambiguous 3D reference frame for all particles oriented in this manner (Michael Radermacher, a student of Walter Hoppe who had worked on the problem of reconstruction from projections with regular conical geometry, joined my group as a postdoctoral fellow in 1983).

The concept and measurement of resolution were new terrain, as well, leading to controversies that are still not entirely settled (see [32]). Again the difficulty lies in the fact that a signal is unrecognizable against the noise background, unlike the situation in crystals where sharp spots mark the extent of the signal in reciprocal space. In two-dimensional averaging, differential phase residual [13] and Fourier ring correlation [27,33] were introduced as measures of reproducibility for half-set averages. The generalization of these measures to three dimensions later on was straightforward.

The uneven, random distribution of projection directions characteristics for single-particle data collection also required a novel approach to 3D reconstruction via weighted back-projection as the weights were no longer uniform but tied to the precise overall geometry [34]. Key contributions were made toward the development of additional tools of 2D and 3D processing by Jose Maria Carazo and Pawel Penczek, who joined my group as postdoctoral fellows in the mid-1980 s and early-1990 s, respectively. These contributions included the capability to match density maps in 3D [35], the introduction of 3D projection alignment and angular refinement [36] and the use of Wiener filtering in merging data with different contrast transfer functions [37,38] (for a review, see [39]). First reconstructions making use of all these capabilities resulted in a breakthrough in the visualization of the *E. coli* ribosome [40,41], ribosomes in different binding states and various other large molecules including ryanodine receptor, chaperones and hemocyanins (e.g. [42–48]).

In the course of those early years, many mathematical concepts and computational procedures were developed, by my group and others, for recovering structures of molecules lacking symmetry and order. These concepts that are nowadays a staple of the single-particle approach were brought together and expounded in a book [49,50]. In addition to SPIDER [15,51], several additional image processing packages became vehicles of dissemination for a growing stock of programs with increasing sophistication (EMAN [52]; Frealign [38]; IMAGIC [53]; XMIPP [54]).

After the widespread initial skepticism of crystallographically oriented practitioners of cryo-EM was dispelled, the single-particle approach gained large momentum and was picked up by many groups. Richard Henderson's important contribution [55] was the investigation of statistical requirements of image alignment and 3D reconstruction in the single-particle approach for different types of radiation. This study may be seen as an in-depth follow-up of the initial estimation by Saxton and Frank [10]. However, the findings of Henderson's article – the ability, in principle, to obtain atomic resolution for a 100 kD molecule using only 10 000 single-particle projections, stood in sharp contrast with the reality: 3600 particles brought a meager 25 Å resolution for the 2.3 MDa bacterial ribosome [40], and a later 11.5 Å structure of the ribosome required a total of 73 000 particles [56]. The explanation for this large discrepancy was that resolutions in the actual experiments were limited by the modulation transfer functions of the suboptimal recording media available at the time – film and charge-coupled device camera.

Still, in the course of the years leading up to the introduction of the new digital cameras, numerous structures were visualized, among these ribosomes, chaperonins, proteasomes, RNA polymerase and ATP synthases. At this point, it is important to emphasize the role of instrument automation in the adoption of the technology as larger numbers of particles had to be collected in efforts to improve resolution. Carragher and Potter early on recognized the importance of automation in data collection and the need for quality control [57]. It is impossible at this place to enumerate the many reviews written over the years that celebrated the advent of the new technique and its impact on biology – even at the stage of low-resolution 'blobology', let alone to list the original articles, but it is worth mentioning a few most recent reviews that brought in some wider perspective [58–60].

The ribosome played a particularly important role both as test object and poster child of single-particle cryo-EM as it was the first molecular machine exhaustively studied by these means and has been visualized in multiple binding configurations and conformational states by several groups (see reviews by [58,61–63]). Especially in this context, we can see the recent emergence of ribosome structures in multiple states with close-to-atomic resolution (e.g. [64–68]) as emblematic for the progress of the entire field.

One of the most intriguing and promising aspects of single-particle cryo-EM is the ability of the technique to visualize an entire spectrum of states, all coexisting in the same sample ('Story in the Sample' [69]). With increasing resolution, it became evident that the molecule populations in the sample

were actually mixtures, either because of conformational or compositional heterogeneity. Again the ribosome provided the most interesting case studies as it goes through a cycle of processive binding events and conformational changes for each amino acid to be added to the growing polypeptide. It should be noted that even before widespread use of maximum likelihood methods, fast computational sorting via multireference methods provided a plethora of information on the functional dynamics of the ribosome during translation (e.g. [70–73]).

Maximum likelihood methods were first introduced into EM image analysis by Sigworth [74] as a means of improving the accuracy and efficacy of alignment of a set of images. The introduction of maximum likelihood methods to tackle classification of images from heterogeneous single particles by Sjors Scheres in the Carazo group in Madrid [75] and later, in a variant developed at the LMB/MRC in Cambridge ('Relion' [76]), opened up the field to a rigorous approach to computational sorting that is now widely used.

In 2009 I wrote a review [77] looking back on 30 years of methods development in single-particle electron microscopy, taking the first proof-of-concept study [14] as a start. I concluded the review at the time by discussing the prospects of single-particle cryo-EM, but also stating the difficulties one faces in attempting to reach atomic resolution for asymmetric structures. There was every indication, including Richard Henderson's earlier statistical analysis [55], that this goal could in principle be reached with a better recording medium. Without it, though, the signal-to-noise ratio was known to fall off rapidly in Fourier space, and the collection of vast amounts of data seemed the only possible path to recover signal at the highest resolution, by means of averaging.

Just at the time I published that review, the first complementary metal-oxide-semiconductor direct electron detection devices were being tested. This truly transformative technology was developed in parallel at the Laboratory of Molecular Biology/MRC (1), UCSF (2) and UCSD in close liaison with commercial companies, FEI (Portland, OR), GATAN (Pleasanton, CA) and Direct Electron (San Diego, CA), respectively. In 2012, such cameras became commercially available. Compensation of beam-induced motion even at the level of single molecules became a reality [78]. It is certainly justified to call these detectors 'game changers' [79] in the grand game of Structural Biology. At once – but by virtue of basically the same concepts that took 30 years to develop – atomic structures have now come into view, and virtually overnight single-particle cryo-EM has become a serious competitor of X-ray crystallography (e.g. [64,65,67,68,80,81]). As spectacularly demonstrated by Yifan Cheng's group at UCSF, even the structures of small membrane channels can now be solved by single-particle cryo-EM at close-to-atomic resolution [82,83].

It is the sky, no doubt, that the 1976 Science Research News article was talking about.

## Acknowledgements

The author thanks Venki Ramakrishnan for valuable comments and suggestions, and Ming Sun for assistance with the preparation of the manuscript.

## Funding

This work was supported by HHMI and NIH R01 GM 29169.

## References

1. McMullan G, Chen S, Henderson R, Faruqi A R (2009) Detective quantum efficiency of electron area detectors in electron microscopy. *Ultramicroscopy* 109: 1126–1143.
2. Li X, Mooney P, Zheng S, Booth C R, Braunfeld M B, Gubbens S, Agard D A, Cheng Y (2013) Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat. Methods* 10: 584–590.
3. De Rosier D J, Klug A (1968) Reconstruction of three dimensional structures from electron micrographs. *Nature* 217: 130–134.
4. Erickson H P, Klug A (1970) The Fourier transform of an electron micrograph: effects of defocussing and aberrations, and implications for the use of underfocus contrast enhancement. *Ber. Bunsenges. Phys. Chem.* 74: 1129–1137.
5. Unwin P N, Henderson R (1975) Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* 94: 425–440.
6. Crowther R A, Amos L A, Finch J T, De Rosier D J, Klug A (1970) Three dimensional reconstructions of spherical viruses by Fourier synthesis from electron micrographs. *Nature* 226: 421–425.
7. Frank J (1975) Averaging of low exposure electron micrographs of non-periodic objects. *Ultramicroscopy* 1: 159–162.
8. Robinson A L (1976) Electron microscopy: imaging molecules in three dimensions. *Science* 192: 360–400.
9. Henderson R, Unwin P N T (1975) Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* 257: 28–32.
10. Saxton W O, Frank J (1976) Motif detection in quantum noise-limited electron micrographs by cross-correlation. *Ultramicroscopy* 2: 219–227.
11. Frank J (1976) Proceedings of the Sixth European Congress on Electron Microscopy, Jerusalem, September 1976. Y. Ben-Shaul, editor. Tal International Publishing Co., Israel. Vol. I, 273–275.
12. Glaeser R M (1971) Limitations to Significant Information in Biological Electron Microscopy as a Result of Radiation Damage. *J. Ultrastruct. Res.* 36: 466–482.
13. Frank J, Verschoor A, Boublik M (1981) Computer averaging of electron micrographs of 40S ribosomal subunits. *Science* 214: 1353–1355.
14. Frank J, Goldfarb W, Eisenberg D, Baker T S (1978) Reconstruction of glutamine synthetase using computer averaging. *Ultramicroscopy* 3: 283–290.
15. Frank J, Shimkin B, Dowse H (1981) Spider—a modular software system for electron image-processing. *Ultramicroscopy* 6: 343–357.
16. Dubochet J, Lepault J, Freeman R, Berriman J A, Homo J C (1982) Electron-microscopy of frozen water and aqueous-solutions. *J. Microsc.* 128: 219–237.

17. Taylor K A, Glaeser R M (1976) Electron microscopy of frozen hydrated biological specimens. *J. Ultrastruct. Res.* 55: 448–456.
18. Frank J (1973) The envelope of electron microscope transfer functions for partially coherent illumination. *Optik* 38: 519–536.
19. Wade R H, Frank J (1977) Electron microscope transfer function for partially coherent axial illumination and chromatic defocus spread. *Optik* 49: 81–92.
20. Spence J C H (1988) *Experimental High-Resolution Electron Microscopy* (Oxford University Press, New York).
21. Wade R H (1992) A brief look at imaging and contrast transfer. *Ultramicroscopy* 46: 145–156.
22. Fujiyoshi Y, Mizusaki T, Morikawa K, Yamagishi H, Aoki Y, Kihara H, Harada Y (1991) Development of a superfluid helium stage for high-resolution electron microscopy. *Ultramicroscopy* 38: 241–251.
23. Langer R, Frank J, Feltynowski A, Hoppe W (1970) Anwendung des Bilddifferenzverfahrens auf die Untersuchung von Strukturänderungen dünner Kohlefolien bei Elektronenbestrahlung [Application of the image subtraction method on the study of structural changes in thin carbon films upon electron radiation]. *Ber. Bunsenges. Phys. Chem.* 74: 1120–1126.
24. van Heel M, Frank J (1981) Use of multivariate statistics in analysing the images of biological macromolecules. *Ultramicroscopy* 6: 187–194.
25. Mossman K (2007) Profile of Joachim Frank. *Proc. Natl. Acad. Sci. USA* 104: 19668–19670.
26. Frank J, Verschoor A, Boublik M (1982) Multivariate statistical analysis of ribosome electron micrographs. *J. Mol. Biol.* 161: 107–133.
27. van Heel M, Keegstra W, Schutter W, Van Bruggen E F J (1982) In: Wood E J (ed.), *EMBO Workshop*, Vol. 1, pp. 69–73 (Harwood Academic Publishers, Chur).
28. Radermacher M, Wagenknecht T, Verschoor A, Frank J (1986) A new 3-D reconstruction scheme applied to the 50S ribosomal subunit of E. coli. *J. Microsc.* 141: RP1–RP2.
29. Radermacher M, Wagenknecht T, Verschoor A, Frank J (1987) Three-dimensional reconstruction from a single-exposure, random conical tilt series applied to the 50S ribosomal subunit of *Escherichia coli*. *J. Microsc.* 146: 113–136.
30. van Heel M (1987) Angular reconstitution: a posteriori assignment of projection directions for 3D reconstruction. *Ultramicroscopy* 21: 111–123.
31. Goncharov A B, Vainshtein B K, Ryskin A I, Vagin A A (1987) Three-dimensional reconstruction of arbitrarily oriented particles from their electron photomicrographs. *Sov. Phys. Crystallogr.* 32: 504–509.
32. Penczek P A (2010), Resolution measures in molecular electron microscopy. In: Jensen G J (ed.), *Methods in Enzymology*, pp 73–100 (Academic Press, Elsevier, San Diego).
33. Saxton W O, Baumeister W (1982) The correlation averaging of a regularly arranged bacterial cell envelope protein. *J. Microsc.* 127: 127–138.
34. Radermacher M (1988) Three-dimensional reconstruction of single particles from random and nonrandom tilt series. *J. Electron. Microsc. Tech.* 9: 359–394.
35. Carazo J M, Frank J (1988) Three-dimensional matching of macromolecular structures obtained from electron microscopy: an application to the 70S and 50S *E. coli* ribosomal particles. *Ultramicroscopy* 25: 13–22.
36. Penczek P A, Grassucci R A, Frank J (1994) The ribosome at improved resolution: new techniques for merging and orientation refinement in 3D cryo-electron microscopy of biological particles. *Ultramicroscopy* 53: 251–270.
37. Penczek P, Zhu J, Schröder R, Frank J (1997) Three dimensional reconstruction with contrast transfer compensation from defocus series. *Scanning Microsc.* 11: 147–154.
38. Grigorieff N (1998) Three-dimensional structure of bovine NADH:ubiquinone oxidoreductase (complex I) at 22 A in ice. *J. Mol. Biol.* 277: 1033–1046.
39. Penczek P A (2010) Chapter two-image restoration in cryo-electron microscopy. *Methods Enzymol.* 482: 35–72.
40. Frank J, Zhu J, Penczek P, Li Y, Srivastava S, Verschoor A, Radermacher M, Grassucci R, Lata R K, Agrawal R K (1995) A model of protein synthesis based on cryo-electron microscopy of the *E. coli* ribosome. *Nature* 376: 441–444.
41. Stark H, Mueller F, Orlova E V, Schatz M, Dube P, Erdemir T, Zemlin F, Brimacombe R, Vanheel M (1995) The 70s *Escherichia coli* ribosome at 23 angstrom resolution: fitting the ribosomal RNA. *Structure* 3: 815–821.
42. Agrawal R K, Penczek P, Grassucci R A, Frank J (1998) Visualization of elongation factor G on the Escherichia coli 70S ribosome: the mechanism of translocation. *Proc. Natl. Acad. Sci. USA* 95: 6134–6138.
43. Beckmann R, Bubeck D, Grassucci R, Penczek P, Verschoor A, Blobel G, Frank J (1997) Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. *Science* 278: 2123–2126.
44. Lambert O, Boisset N, Penczek P, Lamy J, Taveau J C, Frank J, Lamy J N (1994) Quaternary structure of Octopus vulgaris hemocyanin. Three-dimensional reconstruction from frozen-hydrated specimens and intramolecular location of functional units Ove and Ovb. *J. Mol. Biol.* 238: 75–87.
45. Orlova E V, Dube P, Harris J R, Beckman E, Zemlin F, Markl J, vanHeel M (1997) Structure of keyhole limpet hemocyanin type 1 (KLH1) at 15 angstrom resolution by electron cryomicroscopy and angular reconstitution. *J. Mol. Biol.* 271: 417–437.
46. Radermacher M, Wagenknecht T, Grassucci R, Frank J, Inui M, Chadwick C, Fleischer S (1992) Cryo-EM of the native structure of the calcium release channel/ryanodine receptor from sarcoplasmic reticulum. *Biophys. J.* 61: 936–940.
47. Roseman A M, Chen S, White H, Braig K, Saibil H R (1996) The chaperonin ATPase cycle: mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell* 87: 241–251.
48. Stark H, Rodnina M V, Rinke-Appel J, Brimacombe R, Wintermeyer W, van Heel M (1997) Visualization of elongation factor Tu on the *Escherichia coli* ribosome. *Nature* 389: 403–406.
49. Frank J (1996) *Three-Dimensional Electron Microscopy of Macromolecular Assemblies*, pp 12–53 (Academic Press, San Diego).
50. Frank J (2006) *Three-dimensional Electron Microscopy of Macromolecular Assemblies: Visualization of Biological Molecules in Their Native State*. 2nd edn (Oxford University Press, New York).
51. Frank J, Radermacher M, Penczek P, Zhu J, Li Y, Ladjadj M, Leith A (1996) SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J. Struct. Biol.* 116: 190–199.

52. Ludtke S J, Baldwin P R, Chiu W (1999) EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* 128: 82–97.
53. van Heel M, Harauz G, Orlova E V, Schmidt R, Schatz M (1996) A new generation of the IMAGIC image processing system. *J. Struct. Biol.* 116: 17–24.
54. Marabini R, Masegosa I M, San Martin M C, Marco S, Fernandez J J, de la Fraga L G, Vaquerizo C, Carazo J M (1996) Xmipp: an image processing package for electron microscopy. *J. Struct. Biol.* 116: 237–240.
55. Henderson R (1995) The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules. *Q. Rev. Biophys.* 28: 171–193.
56. Gabashvili I S, Agrawal R K, Spahn C M T, Grassucci R A, Svergun D I, Frank J, Penczek P (2000) Solution structure of the *E. coli* 70S ribosome at 11.5 angstrom resolution. *Cell* 100: 537–549.
57. Potter C S, Chu H, Frey B, Green C, Kisseberth N, Madden T J, Miller K L, Nahrstedt K, Pulokas J, Reilein A, et al. (1999) Leginon: a system for fully automated acquisition of 1000 electron micrographs a day. *Ultramicroscopy* 77: 153–161.
58. Binshtein E, Ohi M D (2015) Cryo-electron microscopy and the amazing race to atomic resolution. *Biochemistry* 54: 3133–3141.
59. Cheng Y, Grigorieff N, Penczek P A, Walz T (2015) A primer to single-particle cryo-electron microscopy. *Cell* 161: 438–449.
60. Nogales E, Scheres S H (2015) Cryo-EM: a unique tool for the visualization of macromolecular complexity. *Mol. Cell* 58: 677–689.
61. Frank J, Spahn C M T (2006) The ribosome and the mechanism of protein synthesis. *Rep. Prog. Phys.* 69: 1383–1417.
62. Stark H (2002) Three-dimensional electron cryomicroscopy of ribosomes. *Curr. Protein. Pept. Sci.* 3: 79–91.
63. Valle M (2011) Almost lost in translation. Cryo-EM of a dynamic macromolecular complex: the ribosome. *Eur. Biophys. J.* 40: 589–597.
64. Amunts A, Brown A, Bai X C, Llacer J L, Hussain T, Emsley P, Long F, Murshudov G, Scheres S H, Ramakrishnan V (2014) Structure of the yeast mitochondrial large ribosomal subunit. *Science* 343: 1485–1489.
65. Li W, Liu Z, Koripella R K, Langlois R, Sanyal S, Frank J (2015) Activation of GTP hydrolysis in mRNA-tRNA translocation by elongation factor G. *Sci. Adv.* 1: e1500169.
66. Sun M, Li W, Blomqvist K, Das S, Hashem Y, Dvorin J D, Frank J (2015) Dynamical features of the *Plasmodium falciparum* ribosome during translation. *Nucleic Acids Res.*, doi: 10.1093/nar/gkv991.
67. Wong W, Bai X C, Brown A, Fernandez I S, Hanssen E, Condron M, Tan Y H, Baum J, Scheres S H (2014) Cryo-EM structure of the *Plasmodium falciparum* 80S ribosome bound to the anti-protozoan drug emetine. *Elife* 3: e03080.
68. Fischer N, Neumann P, Konevega A L, Bock L V, Ficner R, Rodnina M V, Stark H (2015) Structure of the *E. coli* ribosome-EF-Tu complex at <3 Å resolution by Cs-corrected cryo-EM. *Nature* 520: 567–570.
69. Frank J (2010) The ribosome comes alive. *Isr. J. Chem.* 50: 95–98.
70. Agirrezabala X, Lei J, Brunelle J L, Ortiz-Meoz R F, Green R, Frank J (2008) Visualization of the hybrid state of tRNA binding promoted by spontaneous ratcheting of the ribosome. *Mol. Cell.* 32: 190–197.
71. Budkevich T, Giesebrecht J, Altman R B, Munro J B, Mielke T, Nierhaus K H, Blanchard S C, Spahn C M (2011) Structure and dynamics of the mammalian ribosomal pretranslocation complex. *Mol. Cell.* 44: 214–224.
72. Fischer N, Konevega A L, Wintermeyer W, Rodnina M V, Stark H (2010) Ribosome dynamics and tRNA movement by time-resolved electron cryomicroscopy. *Nature* 466: 329–333.
73. Ratje A H, Loerke J, Mikolajka A, Brunner M, Hildebrand P W, Starosta A L, Donhofer A, Connell S R, Fucini P, Mielke T, et al. (2010) Head swivel on the ribosome facilitates translocation by means of intra-subunit tRNA hybrid sites. *Nature* 468: 713–716.
74. Sigworth F J (1998) A maximum-likelihood approach to single-particle image refinement. *J. Struct. Biol.* 122: 328–339.
75. Scheres S H W, Gao H X, Valle M, Herman G T, Eggermont P P B, Frank J, Carazo J M (2007) Disentangling conformational states of macromolecules in 3D-EM through likelihood optimization. *Nat. Methods* 4: 27–29.
76. Scheres S H (2012) A Bayesian view on cryo-EM structure determination. *J. Mol. Biol.* 415: 406–418.
77. Frank J (2009) Single-particle reconstruction of biological macromolecules in electron microscopy – 30 years. *Q. Rev. Biophys.* 42: 139–158.
78. Brilot A F, Chen J Z, Cheng A, Pan J, Harrison S C, Potter C S, Carragher B, Henderson R, Grigorieff N (2012) Beam-induced motion of vitrified specimen on holey carbon film. *J. Struct. Biol.* 177: 630–637.
79. Gebel Berg E (2015) Breaking the Crystal Ceiling. *ACS Central Sci.* 1: 283–285.
80. Bai X C, Fernandez I S, McMullan G, Scheres S H (2013) Ribosome structures to near-atomic resolution from thirty thousand cryo-EM particles. *Elife* 2: e00461.
81. Bartesaghi A, Merk A, Banerjee S, Matthies D, Wu X, Milne J L, Subramaniam S (2015) 2.2 A resolution cryo-EM structure of beta-galactosidase in complex with a cell-permeant inhibitor. *Science* 348: 1147–1151.
82. Liao M, Cao E, Julius D, Cheng Y (2013) Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* 504: 107–112.
83. Cao E, Liao M, Cheng Y, Julius D (2013) TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* 504: 113–118.