Nanoliter sample preparation for electron microscopy and single-cell analysis

Abstract

Protein structure determination has long been dominated by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Until recently, electron microscopy (EM) at cryogenic temperatures (cryo-EM) has played a minor role in high-resolution structure determination due to technical reasons. However, with the advent of direct electron detection cameras, and the ability to record high frame rate movies, instead of single long-exposure images, cryo-EM has quickly caught up and is now recognized as a full-fledged method for structural analysis. In contrast to X-ray crystallography, cryo-EM does not require protein crystals, which are difficult, or sometimes even impossible to grow. On the contrary, cryo-EM allows to image individual protein particles in a nearly physiological, frozen-hydrated environment. And unlike NMR spectroscopy, cryo-EM works well with large protein complexes and requires only a few thousand to million particles to be imaged for structural analysis. This allows the structure determination of a protein from extremely low sample volumes. However, EM sample preparation has almost been excluded from the recent advances in the field. It is still dependent on filter paper blotting, a method used to remove excess sample during preparation. This blotting step consumes high amounts of sample, and is often responsible for many problems observed in EM sample preparation, such as reproducibility issues, and loss or degradation of sample. Sample preparation is now widely recognized as the largest bottleneck in the EM structural analysis pipeline.

EM is, in principle, a quantitative and highly sensitive method that can detect single particles and provide structural information in parallel. These qualities can be used for approaches other than structure determination, such as single-cell visual proteomics. Visual proteomics aims at spreading the lysate of a single cell on an electron transparent support and imaging it by EM. Visually distinguishable protein particles are then detected and counted. This, however, requires (i) the lossless preparation of single-cell lysate samples, and (ii) the complete imaging of the prepared sample by EM. Such biological experiments with single-cell resolution have become a major field of research. The main reason for single-cell analysis lies in the heterogeneity of cell populations. Due to the stochastic nature of biological processes, seemingly identical cells can develop different phenotypes. Some of these variations can lead to serious disorders. Tumor heterogeneity, for example, is limiting the efficiency of medical treatments. And the selective vulnerability of certain neurons could be the basis of many neurodegenerative diseases.

A main goal of this thesis was to extend single-cell analysis to electron microscopy, thus enabling future visual proteomics studies. The major work consisted of developing novel EM sample preparation methods. The focus was laid on minimum sample volume requirements and lossless preparation. Both are a prerequisite for single-cell analysis by electron microscopy.

First, a single-cell lysis instrument was built that allowed live-cell imaging and targeted lysis of individual cells from a mammalian tissue culture through a microcapillary electrode. Subsequently, liquid handling was continually improved, until sample volumes as low as three nanoliters could be controlled by the instrument. Such low volumes demanded new approaches

for EM sample preparation. Nanoliter sample conditioning inside a microcapillary tip was developed to transport negative stain in, and salt ions out of the sample plug by diffusion. With this method, nanoliter samples of protein particles, protein nanocrystals, and single-cell lysate were successfully prepared for negative stain EM. To benefit from the most recent developments in cryo-EM, including high-resolution imaging, the instrument was further developed to perform cryogenic sample preparation. Therefore, a dew point stage and plunge-freezing mechanism was invented. The invention allowed to control the temperature of the EM grid, to apply a thin sample film, estimate its thickness through an optical detection, and to quickly plunge-freeze the sample for vitrification. A 5 Å structure of the protein urease was solved by collecting a few thousand imaged particles, prepared from 20 nanoliters of sample.

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* *Authors contributed equally*

Patents

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