Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

Expanding horizons of cryo-tomography to larger volumes

Michael Elbaum

The three dimensional ultrastructure of cells and tissues comes to light with tomography. There is an inherent tension between representing molecular detail at the highest possible resolution, on one hand, and visualizing spatial relations between large organelles or even neighboring cells in large volumes, on the other. Together with its advantages for pristine sample preservation, cryo-tomography brings particular constraints. A major challenge has been the restriction to specimens thinner than the typical cell. New imaging modalities are now available to extend cryo-tomography to thicker specimens: cryo-scanning transmission electron tomography (CSTET), soft X-ray tomography (SXT), and serial surface imaging using the focused ion beam — scanning electron microscope (FIB-SEM). Each one offers specific advantages so the optimal choice depends on priorities among resolution, composition, and volume.

Address
Dept of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot 7610001, Israel

Corresponding author: Elbaum, Michael (michael.elbaum@weizmann.ac.il)

Current Opinion in Microbiology 2018, **43**:155–161

This review comes from a themed issue on **The new microscopy**

Edited by **Stephan Uphoff** and **Ariane Briegel**

https://doi.org/10.1016/j.mib.2018.01.001

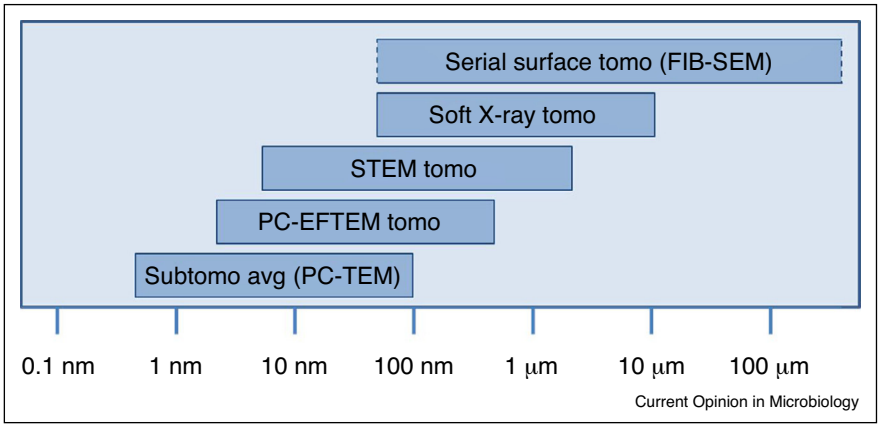
Introduction

Three-dimensional imaging of cells and cell-cell interactions offers a powerful analysis of biological function. Until not so long ago, a frustrating truism held that most features of interest are smaller than the resolution limits of the light microscope, but larger than what can be seen intact in the electron microscope. The past decade has seen a number of new approaches emerge and reach the mainstream. Abbe's resolution limit in the light microscope has been surpassed, at least for fluorescence imaging, and ultrastructure is no longer synonymous with ultrathin sections. The electron microscope is commonly used for imaging in 3D. Tilt tomography is common in transmission EM, and this is complemented by 'serial surface' imaging in scanning EM. Cryo-microscopy based on vitrification of the water medium, an approach developed for macromolecules, has expanded to study of cells. Still, the first litmus test for electron tomography is the frustratingly technical question: how thick is the specimen?

The 2017 Nobel Prize in Chemistry was awarded to pioneers of cryo-EM. The advantages of cryogenic fixation are easy to enumerate. When successful, the sample structure is preserved as a snapshot in time. Nothing is removed, in particular water, and nothing is added, so the chemical composition is also perfectly preserved. The catch is that the water must not crystallize. The need to vitrify already limits the thickness of the specimen. The common method of plunging to liquid ethane relies on rapid removal of heat so that the water molecules arrest in place. When the film is too thick, removal of heat is slow and the center of the film crystallizes. Plunging is generally reliable up to a thickness of about 3 microns. The alternative is high pressure freezing. Water is an unusual material in that it melts under pressure, so rapid cooling under pressure circumvents crystallization. Specimens even 200 microns thick can be vitrified this way.

Presently there are four cryo-microscopy approaches to study cellular ultrastructure in 3D: a) energy-filtered cryo-electron tomography based on phase contrast, with extensions using the Volta phase plate for contrast and focused ion milling to thin the sample, b) all-cryogenic focused ion beam — scanning EM 'serial surface' tomography, c) soft X-ray cryo-tomography (SXT), and d) cryo-scanning transmission electron tomography (CSTET). Each of these methods holds specific advantages so there is no single best choice. The following short review summarizes the relative strengths of each. Generally, access to larger 3D volumes implies a compromise in the attainable resolution, as shown in Figure 1. Three of the methods involve tilt tomography, where the raw data are collected as a series of images as the sample is rotated around an axis. Each image represents a projection through the specimen, and reconstruction converts the series of projections into a Cartesian volume. Serial surface imaging uses either an ion beam or microtome mounted inside the scanning EM to abrade a block of material while iteratively imaging the freshly exposed surface. While both methods were initially developed for plastic-embedded material, ion beam milling has been adapted for 3D cryo-imaging as well.

Figure 1



Cryo-microscopy spans five orders of magnitude in size, from Angstroms to fractions of a millimeter. Different approaches are best suited to different size realms. Boxes depict these realms, with the left edge representing maximal resolution and the right edge maximal thickness according to approximate physical scales explained in the text. For serial surface tomography these scales are not yet entirely clear (dashed lines). PC-TEM: phase contrast TEM; PC-EFTEM: phase contrast energy filtered TEM; STEM tomo: cryo-scanning transmission electron tomography (CSTET); SXT, soft X-ray tomography; FIB-SEM: focused ion beam — scanning electron tomography.

Phase contrast TEM with new improvements

When the highest possible resolution is top priority, there is no match for phase contrast transmission EM [1–3]. The phase image is highly sensitive to small changes in density, but contrast is obtained by taking the sample out of focus, typically by several microns. The image then relates in a complicated way to the density in the specimen. Correcting the contrast transfer function (CTF) properly was key to reaching high resolution for macromolecular structures. The typically flat background in cryo-EM, for example, is an artifact of the CTF. For tomography the correction is complicated due to the change in defocus across the sample for each tilt angle, as well as the sample thickness [4–6]. With sufficiently elaborate CTF correction and sub-tomogram averaging, tomographic data can provide resolution comparable with single particle methods [7,8,9•]. Recent developments in phase plate technology using the Volta effect provide contrast with minimal defocus [10]. This greatly simplifies the CTF and image interpretation [11]. In the short time since it became available, Volta phase plate tomography has been used to study membrane fusion events in lipid bilayers induced by influenza virus haemagglutinin [12••], by mammalian membrane attack complex structures [13,14•], and by neuronal synaptic proteins [15•]. Moving to intact cells, phase plate tomography showed the localization and conformational state of proteasomes and tripeptidyl peptidase II in intact hippocampal neurons [16,17]. A common theme in these works is the benefit of the improved contrast to study heterogeneous structures not amenable to averaging. Combining use of the phase plate with preparation of cryo-lamellae in the FIB-SEM [18–20] provides 3D reconstructions of the intracellular environment with stunning contrast and quality [21••].

Phase contrast requires phase coherence. This imposes the main restriction on sample thickness. Illuminating electrons that lose energy to electrons in the specimen can no longer contribute useful contrast. An energy filter is used to block them from reaching the camera but the sample still suffers damage. The characteristic thickness on which half the electrons are lost is about 200 nm. Not surprisingly, this corresponds to the target thickness for sample milling by focused ion beam. A more subtle limitation to phase contrast tomography is the signal linearity. Suppose two identical objects sit side by side and appear in the image with a certain dark intensity. When tilted so that they overlap, the darkness should double in order to satisfy the basic projection assumption for tomography. Linearity is satisfied when phase differences are small, in the 'weak phase object' approximation. Unfortunately the conditions for a bona fide weak phase object are very restrictive, equivalent to about 30–50 nm of protein embedded in water [22,23]. Quantitative contrast at high resolution is really the realm of thin samples.

Serial surface imaging in the cryo-FIB-SEM

At the other extreme, 'serial surface' imaging with the scanning electron microscope has no intrinsic thickness limitation. The scanning electron microscope can reach nm resolution in imaging a sample surface, so the trick is to record multiple surfaces at progressive depths. Such methods include block face imaging with an incorporated microtome [24], array tomography where serial sections are laid out [25], and iterative FIB-SEM tomography [26]. The last has been adapted elegantly to cryo-tomography [27].

High pressure freezing can be used to vitrify hydrated specimens as thick as 200 μm, so the FIB-SEM approach