Making invisible visible – Electron tomography shows a lattice surface layer in whole-mounts of *Lactobacillus brevis*

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Earlier, transmission electron microscopy (TEM) tomography has been a time consuming method that took even months to accomplish. Today, the procedures can be completed in a day. The rapid changes in tomography procedures are principally due to instrumental improvements. First, modern TEM collects image tilt-series directly in digital format, either manually or automatically. Second, computing sufficient-sized tomograms with the modern supercomputers is a reasonably fast procedure. Besides the self-evident benefits that a tomogram will bring to structural studies we want to emphasize surprising facts. Three-dimensional (3D) reconstructions, i.e. tomograms or 3D maps, do not only make visible tiny structures like 1.4 nm gold particles that are almost impossible to detect in original TEM images. In addition, delicate broad frameworks like the surface layer in *Lactobacillus brevis* appear clearly visible in tomograms. In the raw data, i.e. tilt series, indications of a surface layer structure cannot be seen even with enhanced image processing and in stereo viewing. The lattice surface layer that appears in 3D maps using the maximum-entropy method is visualized optimally in stereo. Also solid 3D prints of tomograms are possible to construct easily today, accurately and at high resolution. 3D prints are important in interpreting and comparing 3D maps.

**Keywords** electron microscopy, electron tomography; 3D reconstruction; maximum-entropy method; whole-mounts; 3D prints; Lactobacillus brevis; surface layer; S-layer

1. Introduction

Images acquired with TEM are orthogonal projections of objects under study. Objects from thin sections down to protein samples are fundamentally all three dimensional (3D) structures. Consequently the collected images in TEM are two dimensional (2D) images, in other words only shadows of the original 3D object. Due to superimposing effects, structural details in 2D images are more or less blurred. The high resolution achievable in TEM is mostly lost in blurred images, down to 1/10 or more of the actual resolution that could be achieved.

A simple and partial solution for seeing more details at higher resolution is to use aligned stereo TEM image pairs (e.g. with increments between 5°–10°) using anaglyph stereo methods (e.g. Adobe Photoshop or free ImageJ) using red-cyan or red-green glasses with any ordinary desktop computer. The anaglyph method allows to magnify the anaglyph image without losing the stereo effect. Magnification is not possible with stereo pairs placed side by side at fixed distance (~6.5 cm or less) as they cannot be moved much apart without causing eye strain. TEM stereo pairs do improve resolution. Stereo pair visualizing is limited as it is static and thus immobile. A more dynamic solution can be achieved by collecting tilt-series and visualizing the sequence after alignment as a stereo movie that we regularly perform with our alignment program JPEGANIM in SGI machines [1–3].

3D reconstruction by electron tomography is a more elegant solution [1–3]. Tomograms can be studied from any direction as transparent or in isosurface mode, pixel thin sections can be inspected and picking some peptides from the protein data bank may show up some which fit into the 3D maps. If particles can be purified to a high degree of homogeneity, single particle reconstruction with programs like EMAN [4] is a much better solution as the amount of different views of the object can be increased to 103–5 times and because of this much higher resolution close to atomic resolution can be achieved in theory. Here we will present our electron tomography methods (ETM) [1–3] using whole-mounted critical-point dried (CPD) *Lactobacillus brevis* in which we could see a surface layer clearly with ETM. The surface layer in *L. brevis*, a Gram-positive bacterium, has for some reason been difficult to show with standard methods like negative staining. We have the impression that CPD whole-mount samples have been used less than the cryo-TEM preparations to produce tomograms today. We must recognize that though cryo-TEM samples are closer to native states the signal-to-noise ratios (SNRs) are much less compared to CPD whole-mounts. High SNRs is the presumable the reason that might explain the appearance of the surface layer in tomograms of whole-mounted CPD *L. brevis* preparations. Support for this is the fact that e.g. 1.4 nm immunogold markers, as we have shown [2–3], are clearly visualized in tomograms of...
whole-mounted CPD eukaryotic cells used in immuno-electron tomography (IET), but they are extremely difficult to detect in images of the raw data tilt-series. As our IET methods have recently been published [2, 3] we are not going to present them here.

2. Material and Methods

2.1 Material

*Lactobacillus brevis*, strain ATCC 8287 was used for study the surface layer. The bacteria cells were either (1) intact cells or (2) starved cells, i.e. kept in water at 4°C for 3–5 days.

2.2 Preparation of whole-mounted CPD samples

Detailed protocols have been published [2,3]. Briefly, holey-carbon grids (Quantifoil, Multi A, Au-100x400 mesh) coated with gold markers were used for alignment of the tilt-series [1–3] by dipping both sides of the grid in 2:10 dilution of the 5 or 10 nm commercial immunogold stock solution (e.g. IgG, Gold conjugates, Sigma) with filtered (0.22 µm Millipore) deionized distilled water (fDDW). After application of gold markers the grids were glow-discharged (GD) and 3 µL cell suspension (1:10–100 dilution of the original sample in fDDW) were put on grids. After 0.5–1 minutes the grid samples were floated on drops (~50 µL) of fDDW 5–10 minutes on dimple-Parafilm in a Petri dish as described in [2]. After further washing in fDDW (several drops) cells mounted on the grids were fixed with 1.25% glutaraldhyde (GA) in 0.1M Na-cacodylate (NC), pH 7.4 for ~10–30 minutes with or without sequential staining, after washing in droplets of fDDW, in 1% tannic acid (TA) in fDDW or in a mixture of NC and HEPES+Mg buffer (10 mM MgCl₂ in 10mM Hepes, pH 7.4) 1:1 for 5–10 minutes. Also samples fixed by adding 0.3% OsO₄ (~5 minutes) to 1.25% prefixed GA (10 minutes) with or without sequential staining in 1%TA (as above) were prepared. After a brief washing in fDDW the samples were dehydrated in methanol and stained (30 s) in diluted (0.002%) uranyl acetate (UA) in 100% methanol. After washing in methanol and replacing methanol with acetone the samples were critical-point dried.

2.3 Raw data tilt-series, electron tomography, 3-D visualization and solid 3-D prints

For recording tilt-series 120 kV TEM (FEI, Tecnai 12 BioTwin) and 300 kV TEM (Jeol JEM-3200FSC) were used. With the 300 kV TEM the Omega type energy filter with zero-loss-image mode was crucial but no objective aperture was used to collect tilt-series of ±60° total tilt, with 3° increment, manually or semi automatically using SerialEM [5] in 120 kV TEM. In 3-D reconstruction we used our maximum-entropy method (ETM) and 3D visualization in SGI as described [1–3] and UCSF Chimera [6] with Macintosh computers. Solid 3D prints of the 3D maps were produced using ZPrinter 450 (Z Corporation, Burlington, MA).

3. Results and Discussion

Attempts to visualize the surface-layer of *Lactobacillus brevis*, a Gram-positive bacterium, in electron microscopy using standard negative staining methods e.g. with uranyl acetate or uranyl format have been elusive for unknown reasons. In tilt-series of the whole-mounted CPD *L. brevis* collected samples, only a smooth bacterial surface structure is visible in all fixation procedures used (Fig. 1) with no indication of any periodic surface layer.

![Fig. 1 Image of a tilt-series at 0° of (2) starved cells (more transparent), whole-mounted CPD samples, fixed in GA,Ta, UA. Any surface layer cannot be detected at higher magnification or even with image enhancement in these raw data images. Scale bar 100 nm.](image-url)
In 3D reconstruction of whole-mounted CPD samples of *L. brevis* striped band-like structures are usually seen transversally wrapping the surface roughly perpendicularly to the length axis of the bacterial cell (Fig. 2). Striated bands approximately 80 Å wide are shown. The 80 Å striated bands appear also partly interlaced and arranged into a mesh like framework. The appearance of the surface layer resembles, in a way, the skin of some reptiles or the wrinkles on earthworms. Striated bandwidths of some 80 Å are regularly seen at low magnification e.g. ~3600x (Fig. 2). At higher resolution moiré patterns obscure the surface layer (cf. Fig. 4). The surface pattern is more distinct in a solid 3D print (Fig. 3). Figs. 2 and 3 are images of 3D reconstructions of 8x binning of the original 3D map obtained from tilt-series collected at 28,700x magnification.

**Fig. 2** Red-cyan anaglyph stereo image of 3D reconstruction of 8x binning of the 3D map obtained from tilt-series collected at 28,700x magnification at 120 kV (FEI, Tecnai 12), no objective aperture used. Regular wrinkles roughly 80 Å wide are seen transversally decorating the bacterial surface.
Fig. 3. Solid 3D print of the 8x binned 3D map in Fig. 2. The surface layer (in blue color) of *L. brevis* is supported by a supporting rack that the 3D printer options automatically produce for delicate objects. In 3D prints some details are better accentuated than in the original 3D maps. 3D prints cannot produce transparent objects that 3D maps can easily be shown with various computer visualization programs.

Moiré patterns appear on the surface especially when 3D maps at higher magnification are moved and observed from different angles (Fig. 4). We interpret the moiré patterns as interference from superimposed periodic structures of the bacterial surface layer. Thin thread-like structures (10–15 Å width) are seen but the striated ~80 Å periodicity that is obvious in Figs. 2 and 3 cannot be recognized clearly as in 8x binned 3D maps.
Fig. 4 *L. brevis* surface in 3D map of CPD whole-mount specimen (GA fixed, UA stained). Tilt-series recorded (as described in methods) with 120k TEM, without objective aperture. Moiré patterns are seen as described and explained as interference from superimposed periodic structures of the surface layer. Thin threads of approximately 10–15 Å width are visible on the surface.

Following data revealed a good deal of information of *L. brevis* surface layer. A 3D map obtained from CPD whole-mount samples of *L. brevis* that was fixed and stained with GA, OS, TA, UA (see methods part) was revealing. Due to the extra contrast caused by OS fixation and TA staining and due to the crucial transparency of the raw data tilt-series, it was considered appropriate, for achieving a successful 3D reconstruction with ETM, to use high voltage TEM. Tilt-series were recorded with 300 kV TEM, with no objective aperture but it appeared crucial to use the energy filter in zero-loss-image mode (without this the 3D maps produced were not adequate). In stereo the surface structure looked complicated and difficult to get a comprehensive view of. A reconstructed cell wall part was sporadically chosen from the 3-D map for inspection. In this part not only the whole surface wall was exposed but also a thin tangential-like section of the surface layer was reconstructed (Fig. 5).

In Fig. 5 it is seen that from the thin region distinct aligned ladders or rather parts of a thin lattice or mesh-like network is protruding from the thicker part. Therefore it appeared rather clear that these thin lattices are the building units that interface to form a thicker surface layer. At closer look there do not seem to be other elements involved than an amorphous background (blue in color pictures) that seem to be the peptidoglycan or murein layer part as seen in stereo (Fig. 6 a) and in side view (Figs 6b, c). Side views (Fig. 6 b, c) additionally reveal that the ladders or lattice are building blocks of the actual surface layer. Units in the lattices appear as squares or rectangles depending on their orientation. Frontally the square-like units seem to be not more than about 10–15 Å (Fig. 6a) and in side view (Fig. 6b) of the same thickness 10–15 Å.

Accordingly the surface layer in *L. brevis*, because it is a crystalline structure, is built of 10–15 Å square-like lattices that interlace and pile up into layers of mesh-like structures. In the exceptional case with the thin tangential-like section the surface layer is only 10–15 Å thick (Fig. 6 b that is from the thin region in Fig. 5), but in other places about 40 Å thick. With small angle X-ray scattering (SAXS) we have measured a periodicity of about 80 Å in surface layer samples of *L. brevis* [6]. Accordingly some 4–6 unit layers are needed to build up a self-assembling surface layer mesh. However, it is not clear how higher order structures in the range of 80 Å wide striated bands that appear in 8x binned maps (Fig. 2 and 3) are organized in the surface layer of *S. brevis*. The self-assembling building steps of the surface layer are dynamic processes that 3D maps cannot straightly demonstrate. For this 4D maps, i.e., a sequence of snapshot
3D maps [5], are needed in analogy to what we have presented for living cells [6] using a specially built light microscope.

Crystalline-like lattice squares of 10–15 Å of the surface layer in *L. brevis* seem to be smaller than those earlier reported [7]. This might explain why our attempts using negative stain have been elusive as this technique may not fit for detecting structures of this size range.

Moreover, others may criticize that seeing details of 10–15 Å are beyond the resolution obtainable in tomograms according to thumb rules such as Crowther’s criterion [8]. These rules might fit for sections but be less valid for CPD-whole-mounted specimens (by nature of variable thickness) especially when crystalline-like lattice structures are involved as shown here. The lattice structure appear indeed strikingly different from other present structures and may look somehow artificial. Yet this can be due to inherent crystalline nature of the presented structures. The same structures can be seen in same clarity with other visualizing programs [1–3] besides UCSF Chimera [9] to rule out artifacts that an individual program might produce. Actually the lattice-like network structure described is possible to see with all fixation procedures used including simple GA fixation alone with diluted UA (Fig. 4) in all fixations presented, to rule out any artifacts produced by various fixation procedures. In 120 kV as well as 300 kV TEM the same structures can be seen though in the 300 kV TEM we get the impression that details appear sharper as expected.

![Image](image_url)

**Fig. 5** The upper part of the 3D map in the inset is viewed. The surface layer reconstructed including a tangential-like part at higher magnification is shown with a scale bar 80 Å. Surface layer of a 3D map of CPD whole-mount specimen was fixed and stained with GA, OS, TA, UA as described in methods, tilt-series were recorded with 300 kV, the energy filter with zero-loss-image mode was
essential to get advantageous reconstructions. Details of this region are presented at higher magnification in Fig. 6a and as side view in Fig. 6b.

**Fig. 6** a) Details of Fig. 5 in stereo. Parts of a the thin crystalline lattice-like network. The stereo view shows that the lattice structure is adjacent to a more amorphous layer (blue in color pictures) that is presumably of peptidoglycan, also seen in side view in b) and c). The structural lattices seem to be at least partly a monolayer in frontal view as many clear squares of ~10–15 Å, the size of the lattice, can be distinguished as well as almost perpendicular side views of same thickness are apparent, partly revealing how the 3D mesh of the surface layer might be assembled. In b) the same region is seen as a side view showing that the thickness of a mono lattice layer is approximately same as the size of the lattice squares ~10–15 Å. c) Side view from a different region showing a lattice structure of multi-layered thickness of around 40 Å.

**References**


