Tomography and Subtomogram Averaging

John Briggs
What do we need to get a 3D structure?

Sample preparation methods

A transmission electron microscope

Different views of our object of interest

Computational approaches for producing a 3D reconstruction from 2D projections

Methods for validation and interpretation of the 3D structure
What do we need to get a 3D structure?

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Different views of our object of interest

Computational approaches for producing a 3D reconstruction from 2D projections

Methods for validation and interpretation of the 3D structure
We need different views of our object
Tomography?

In single-particle methods we obtain different views of our object of interest by imaging many different copies that are oriented differently relative to the electron beam.

In tomography we obtain different views by physically rotating the sample in the microscope
Movie from the Baumeister lab, Max-Planck Institute for Biochemistry
Tilt series and tomogram

Tilt-series

Tomogram
Why do tomography?

Because our sample is a unique structure (bits of cells, tissues, viruses etc.)

Because our sample is within a complex environment
Topics to be covered

Sample preparation methods

Data collection and microscope requirements

Alignment and reconstruction

Subtomogram averaging
Sample preparation

Sample must have structure preserved

Sample must be thin

Sample must be stable in vacuum
Cellular sample preparation methods

- Chemical fixation
  - Dehydratation
    - Resin embedding
      - Sectioning
        - Cryo-sectioning
          - Immuno-labelling
            - TEM
  - Cryo-fixation (high pressure freezing or plunge freezing)
    - Freeze-substitution / fixation
      - LT-Embedding
        - Sectioning
          - Staining (UA,LC)
            - Cryo-sectioning
              - Cryo-TEM
      - CEMOVIS
        - FIB milling
  - Cryo-sectioning
    - TEM
  - TEM
Cryo-sectioning (CEMOVIS)

High-pressure freezer

Cryo-microtome
The end speed should be set to 204 mm/s and adjust according to the gliding behavior of the sections and personal skill. The strength of the ionizer depends on the setting of the control panel and on the distance of the electrode to the knife edge. If the ionizer produces too much charge the sections will fly away from the knife edge. If the charge is too low the sections will not stretch or will stick to the knife edge. We recommend starting with maximum power to avoid contamination of the knife by sticking sections and then reduce the setting or increase the distance if the sections fly away.

6. Sectioning and transfer of the sections to the grid. Start the automatic sectioning and have the eyelash ready to hold the forming section. Under good conditions the sections will form a ribbon and the ribbon will easily slide off the knife edge. Hold the front of the ribbon above the grid from underneath using the eyelash (see Fig. 7a). When the ribbon is long enough stop automatic sectioning, turn off the ionizer, slightly pull the ribbon and place it on the grid. Detach the ribbon from the knife edge with the eyelash.

See Table 1 that addresses frequent sectioning difficulties (see Note 7).

Fig. 7 Sectioning and transfer of the sections. (a) To the first section of the forming ribbon the eyelash is attached and with it the ribbon is pulled over the grid, which is positioned by the grid holder (GH) close to the knife. The insert shows a higher magnification. (b) The grid with the ribbon on it is pressed between the two porcelain parts of the pressing tool. To apply force the precooled Allen key (AK) is used. Note that the whole grid is not in the pressing tool. The border of the grid out of the pressing tool makes pick up of the grid with the forceps after pressing easier. (c) With the precooled forceps the grid is transferred from the lower part (LP) of the pressing tool into the cryo-grid box (GB) next to the pressing tools (insert).

Artefacts due to cutting

Petr Chlanda
Sample preparation - FIBSEM

Rigort and Plitzko 2015
Sample preparation - FIBSEM

Mahamid et al, Science 2015
Data collection

1. Set new tilt angle
2. Track – correct for shifts
3. Focus
4. Exposure

Baumeister et al. Trends in Cell Biology 1999
Microscope requirements
(for cryo-electron tomography of thicker samples)

High-voltage (ideally 300kV)

Energy filter

Very stable and well-calibrated microscope stage

Appropriate software
Tilt series and tomogram

Tilt-series

Tomogram
Forward and back projection

3D object -> 2D projections

2D projections -> 3D reconstruction
The data is incomplete: the missing wedge

2D projections -> 3D reconstruction

Missing information
The missing wedge leads to smearing in $z$
Crowther Criterion

$$r = \frac{\pi D}{N}$$

$r$: Resolution limit
$N$: Number of projections
$D$: Object thickness

Missing information due to discrete sampling
Data collection

Set new tilt angle

Track – correct for shifts

Focus

Exposure

What questions should we ask before data collection?
Data collection

What total dose? Resolution vs signal-to-noise

What tilt range? Completeness of information vs dose and speed

What angular increment? Resolution vs dose and speed

What order to collect the images? Speed, reliability, optimal dose, sample distortion...

What magnification? Resolution and DQE vs field of view

What defocus? High-frequency information vs low frequency information
Alignment and Reconstruction

Once we have collected the data how do we reconstruct a tomogram?
Alignment and Reconstruction

We need to know how the projections relate to each other: the angles and shifts between the projections.

We have defined the angles in the microscope by tilting around a defined axis by defined increments.

Alignment is necessary to deal with the shifts in the image. (at larger fields of view, other distortions may become important)
Alignment and Reconstruction

- Reconstruction by weighted back projection
Back-projection
Back-projection
Back-projection
Software

Data Collection: **SerialEM**, FEI Tomo, Leginon...

Tomogram reconstruction: **IMOD**, TOM, protomo, PyTOM...
Subtomogram averaging
Subtomogram averaging

Mahamid et al, Science 2015
Subtomogram averaging

Figure 1: Subtomogram averaging process.

1. Tomogram (randomly oriented)
2. Subtomograms (aligned to reference)
3. Averaged subtomograms (new reference)

Use a new reference for alignment and iterate until the reference is stable.
Subtomogram averaging process

Data

CTF correction
Align tilt-series
Reconstruct tomogram
Extract subtomograms

Assign initial Euler angles (priors)
Align subtomograms (3D)
Classify subtomograms
Average subtomograms
Post-processing

Structure
Software for subtomogram averaging

Dynamo (Castano-Diez, Basel)

PEET (Heumann and Mastronarde, Boulder)

PyTOM (Foerster, Utrecht)

RELION (Bharat and Scheres, LMB)

Maximum cross-correlation or Maximum likelihood
Subtomogram averaging

Why not just do single particle reconstruction? (if you can, then do it!)

Subtomogram averaging allows structures to be determined when other objects in the path of the electron beam would otherwise prevent alignment.

(and has potential for application in single-particle type projects)
Subtomogram averaging

Can generate two kinds of information

Structure

Position and context
Subtomogram averaging - challenges

Sample flexibility and heterogeneity

Higher apparent sample thickness (especially at tilt)

Two alignment and reconstruction steps

Sample movement/change during data collection

Higher total electron dose

Smaller datasets (due to more time-consuming data collection and processing)

Difficult in determination and correction of CTF
In which order should we collect the images?
Continuous tilt scheme
Bidirectional tilt scheme
Dose-symmetric tilt scheme
Tilt schemes

Continuous Tilt-Scheme

Bidirectional Tilt-Scheme

Dose-Symmetric Tilt-Scheme

Hagen et al. JSB 2017
Tilt schemes – signal transfer

Note – the difference is greater if you also consider increased sample movement at tilt

Hagen et al. JSB 2017
Dose-dependent sample changes

Hagen et al. JSB 2017
Subtomogram averaging - challenges

CTF Correction
Defocus gradients in the sample

2D CTF correction considers only the gradient due to tilt, 3D CTF also considers the gradient through the thick sample

Turonova. et al
Defocus gradients in the sample

A. Reference

B. No correction

C. 2D-CTF

D. 3D-CTF

Disc #1
x=0nm, z=0nm

Disc #2
x=0nm, z=250nm

Disc #3
x=500nm, z=0nm
Defocus gradients in the sample

Turonova. et al
Subtomogram averaging - challenges

Sample flexibility and heterogeneity

Higher apparent sample thickness (especially at tilt)

Separate alignment and reconstruction steps

Sample movement/change during data collection

Higher total electron dose

Smaller datasets (due to more time-consuming data collection and processing)

Difficult in determination and correction of CTF
It is possible to obtain <4Å resolution

Schur et al. Science 2016
Subtomogram averaging

Structure “in situ”

Cellular context

Structure for “Discovery”

Potential SP applications

Mahamid et al, Science 2015

Mattei et al. Science 2016

and articles by many authors cited therein