Cryo-Applications and sub-tomogram averaging

Tomography Short Course!
4-12-21
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What is CryoET?
(cryo-electron tomography)

• Cells or complex reconstituted environments

Tan ZY et al., bioRxiv 2021
Brasch J et al., Nature 2019
What is CryoET?
(cryo-electron tomography)

>> CryoET is the highest resolution method for native specimen

Tan ZY et al., bioRxiv 2021
Brasch J et al., Nature 2019
Overview – Why CryoET?

Why cryo?
• Specimen preservation in native or near-native environments.

Why electrons?
• +Small wavelengths (high res), +Can be focused, –Damage sample

Why tomography?
• Some combination of:
  o Sample is unique; e.g. cells,
  o Sample is too heterogeneous (structurally or morphologically); e.g. viruses with variable # of receptors, or viruses of different non-symmetric shapes,
  o Domain-stoichiometry and/or orientation is required,
  o Sub-nanometer information is usually not required, but may be possible.
Overview – Why subtomogram averaging?

• Some amount of structural repetition,
• Repeating subunit preferred orientation overcome by tilt range

Courtesy of Misha Kudyashev
Overview

• CryoET limitations
• Tilt-series collection
• Tilt-series alignment
• Defocus estimation and CTF correction
• Sub-tomogram localization
• Sub-tomogram alignment and averaging
• Examples
• Processing limitations
• Future directions and improvements
CryoET Limitations
Overview – Limitations

Limitation: Specimen/Ice thickness

William J. Rice, NYSBC, 2017
300 keV Krios

Vulović, 2013
Overview – Limitations

Limitation: Specimen/Ice thickness

Thompson et al., 2016
Overview – Limitations

Limitation: Camera fidelity

McMullin, 2014 & Ruskin, 2013
Overview – Limitations

Limitation: 

**Electron damage** of the specimen

- High resolution information is lost first.

**Solution:**

Remove damaged information from image frames
Tomography overview
Tomography overview

Tilt-series Collection
Tomography overview
ET/CryoET collection and processing overview

Collect → align → reconstruct

Specimen

-α

+α

tilt-series

0°
3D specimen movement during collection

(movements are exaggerated)
3D specimen movement during collection

(movements are exaggerated)
3D specimen movement during collection

(movements are exaggerated)
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(movements are exaggerated)
3D specimen movement during collection (movements are exaggerated)
3D specimen movement during collection

(movements are exaggerated)
Some more
CryoET Limitations
Grid tilting increases thickness

untilted grid

grid tilted 60° = 2x thickness
Grid tilting thickness increase limits tilting

- Phase plate tilt-series of T20S Proteasome
- Tilt axis is horizontal

Noble et al., eLife 2018
Grid tilting limit results in missing information

Phase plate tilt-series of T20S Proteasome.

Tilt axis is vertical

Noble et al., eLife 2018
Tilt-series collection
Tilt-series collection software

Legion

SerialEM

TOM Toolbox

UCSF Tomography
Automated tilt-series collection is currently routine

- From an atlas, select multiple squares, and from each square select holes,
- For each hole place an exposure target along with one or more focus targets,
- Set up dose, defocus range, tilt model, etc. appropriately,
- Collect!
Automated tilt-series collection

Focus on the tilt axis!

• You want to minimize the amount of tracking error
  ➢ Tilting should not change the x,y,z target location

• This is called getting eucentric height.
Some Collection Schemes

A

Continuous  Bidirectional  Dose-symmetric

Decreasing  Increasing

Turoňová et al., 2019 & 2020
Some Collection Schemes on an *Isotropic* Sample

EMD-4016  Dose-symmetric  Bidirectional  Continuous

Turoňová et al., 2019
Tilt-series alignment
Tilt-series alignment

• **Software:**
  - ETomo in IMOD – **Fiducial-based** alignment (also **patch tracking**)
  - Markerauto and AuTom – Automated **fiducial-based** alignment
  - Protomo – **Fiducial-less** alignment
  - Alignator – **Patch tracking** alignment, GPU-accelerated
  - Dynamo – **Fiducial-based** alignment

• **Must refine** most or all of the following:
  - Tilt image shifts, rotations, defocus changed, & magnification changes
  - Tilt axis location
  - Tilt angles
Fiducial-based tilt-series alignment

- Requires a **sufficient number of well-behaved gold beads**
- Semi-automated (IMOD, Dynamo) or automated (AuTom/markerauto, IMOD) processing

http://bio3d.colorado.edu
Fiducial-based tilt-series alignment issues

Nearby Fiducials Affect Signal and Contrast

- Fiducial fringes change the power spectrum of your reconstructed object.
Fiducial-based tilt-series alignment *issues*

Fiducials are in the reconstruction, *Even if You Can’t See Them!*

- **Distant fiducials** can be in the projection direction of your extracted object of interest.
- Erasing fiducials isn’t perfect.
Identify featureful objects with contrast in all tilt images and track them.

- Semi-automated (IMOD, Alignator)
How does *fiducial-less* alignment in Protomo work?
Collect a tilt-series
Protomo alignment

Nearest-neighbor correlation
Protomo alignment

1. Nearest-neighbor correlation
2. Weighted back-projection
3. Volume to be re-projected
Protomo alignment

Nearest-neighbor correlation
Weighted back-projection
Volume to be re-projected
Re-projection → correlation

$\begin{align*}
\text{alignment thickness} &= z \\
\text{Volume to be re-projected} &\rightarrow \text{correlation}
\end{align*}$
Protomo alignment

1. Nearest-neighbor correlation
2. Weighted back-projection
3. Volume to be re-projected
4. Re-projection → correlation

alignment thickness = z

x y
Protomo alignment

1. Nearest-neighbor correlation
2. Weighted back-projection
3. Re-projection → correlation
4. Volume to be re-projected
5. Weighted back-projection
6. Nearest-neighbor correlation
Protomo alignment

1. Nearest-neighbor correlation
2. Volume to be re-projected
3. Weighted back-projection
4. Re-projection → correlation

\[ x = y = z \]
Protomo alignment

1. Re-projection → correlation
2. Nearest-neighbor correlation
3. Weighted back-projection
4. Volume to be re-projected
5. Re-projection → correlation
6. Nearest-neighbor correlation
7. Weighted back-projection

alignment thickness = z
Protomo alignment

- Weighted back-projection
- Nearest-neighbor correlation

Volume to be re-projected

Re-projection → correlation
Protomo alignment

1. Nearest-neighbor correlation
2. Weighted back-projection
3. Volume to be re-projected
4. Re-projection → correlation
5. Weighted back-projection
6. Nearest-neighbor correlation
7. Weighted back-projection

alignment thickness = z

x

y
Protomo alignment

Nearest-neighbor correlation
Weighted back-projection
Volume to be re-projected
Re-projection → correlation
Protomo alignment

1. Re-projection $\rightarrow$ correlation
2. Volume to be re-projected
3. Weighted back-projection
4. Nearest-neighbor correlation
5. Alignment thickness $= z$
Protomo alignment

2n → 3 → 5 → 4

6 → 7

(x, y) → (z)

- Nearest-neighbor correlation
- Weighted back-projection
- Volume to be re-projected
- Re-projection → correlation
Protomo alignment
Protomo alignment

Refine orientations of objects
Protomo alignment
Protomo alignment

Refine tilt azimuth
Appion-Protomo refinement

Iterate with different filters
Why is this important?
Appion-Protomo refinement

Why is this important?

Iterate with different filters

Nearest-neighbor alignment

After refinement

After refinement
Defocus estimation

**Goal:** Find the **height of your objects** of interest to correct for microscope aberrations (CTF)

**Problem:** Low per-image **SNR** and potential poor tracking

Zhang, 2016

High dose single particle image

3 e^-/Å² single particle tilt image
CTF estimation and correction for tilt-series or tomograms
Defocus estimation methods

Methods ordered approximately \textit{worst-to-best} (depends on sample):

\begin{itemize}
  \item \textbf{Per-image} defocus estimation accounting for tilts (CTFFIND4, GCTF, etc.)
  \item Per-tomogram post-hoc estimation by using \textit{SPT FSC to locate the first CTF zero}
  \item \textbf{Image tiling} to estimate the \textit{defocus of the untilted plane} (TomoCTF)
  \item Defocus estimation and \textit{interpolation using two focus locations} on the tilt axis (Eibauer, 2012)
  \item Per-particle tilt image fine estimation and correction that accounts for the \textit{3D location of each particle}
  \item Per-particle tilt image fine estimation and correction that takes into account \textit{overlapping objects} in each tilt image of each particle and accounts for the \textit{3D location of each particle} – can use all particles in each tilt image to refine!
\end{itemize}
CTF correction methods

Methods ordered approximately **worst-to-best** (depends on sample):

- **Per-image** correction
- Strip-based correction with TomoCTF or IMOD ctfphaseflip
  - Flips phases and optionally corrects amplitudes (TomoCTF) on a strip-by-strip basis.
  - Error will depend on the amount of non-eucentricity
- 3D CTF model (Relion) takes into account x,y,z particle locations
- Per-particle/tiling CTF correction (EMAN2)
- During tomographic reconstruction (EmSART, NovaCTF)
Sub-tomogram processing
Sub-tomogram processing workflow

- **Missing wedge** must be taken into account for each sub-tomogram
Must take into account subtomogram missing wedges

- Effectively align volume in common in Fourier space

Sub-tomogram processing software

- Dynamo – GPU accelerated, tomogram database, extensive picking abilities
- Relion – 3D CTF model, Bayesian approach to alignment is used
- EMAN2 – Sub-tilt-series refinement and defocus estimation/correction
- emClarity – Sub-tilt-series refinement and defocus estimation/correction
- TYGRESS – Intended for use w/ high dose 0 degree image (Nicastro group)
- PyTom
- PEET
- Jsubtomo
- TOM & AV3
- XMIPP
- Warp
Sub-tomogram processing in Relion

- Uses normal Relion workflow.
- Potential issues:
  - Extra images are likely not at the same focus as the Target
  - 3D FSC may eliminate properly interpolated values due to sampling

Bharat et. al., Structure 2015
Sub-tomogram processing in Relion

![Graph showing scaling factor and Applied B-Factor against Tilt angle and Electron dose]
Sub-tomogram processing in Relion

- Test case: Hepatitis B capsid

Bharat et. al., Structure 2015
Sub-tomogram processing in Relion

- 6e-/Å² pre-exposures prior to tilt-series collected were collected and analyzed with single particle
Sub-tomogram processing in EMAN2

Galaz-Montoya, JSB 2016
Sub-tomogram processing in EMAN2

- Better than 2/3 Nyquist
Tomogram annotation
Tomogram/sub-tomogram annotation and segmentation software

- Dynamo – Annotate membranes, tubes, helices, crystal structures, vesicles, etc.
- EMAN2 – Neural network segmentation
- Amira – Interactive segmentation and filtering suite
- UCSF Chimera w/ Segger - Interactive segmentation
- Template picking – MolMatch, Dynamo
- Various deep learning picking and segmentation softwares
Sub-tomogram annotation processing in Dynamo

- Backbone, helical, and circumferential picking
- Helical symmetry determination

Castaño-Díez et. al., JSB 2012 & 2016
Sub-tomogram annotation processing in Dynamo

Castaño-Díez et. al., JSB 2012 & 2016
Sub-tomogram annotation processing in Dynamo

Castaño-Díez et. al., JSB 2012 & 2016
Sub-tomogram segmentation with CNNs in EMAN2

Chen et al., Nat. Meth. 2017
Sub-tomogram segmentation with CNNs in EMAN2

Chen et. al., Nat. Meth. 2017
Template matching

Lucic et al, 2005, Annu. Rev. Biochem
CryoET allows for glimpses into cells/tissues
CryoET allows for glimpses into cells/tissues

Thompson et. al., 2016
CryoET allows for glimpses into cells/tissues

Baumeister et al., MPI
CryoET allows for glimpses into cells/tissues

Schaffer et al., Nat. Meth.
CryoET allows for glimpses into cells/tissues

Kelley et al., 2020
CryoET allows for glimpses into cells/tissues

Kelley et al., 2020
Examples from the literature
Example: STA followed by placing averages to the tomograms

Example: COPII proteins on budding GUVs

Over-picking to find repeating units

Hutchings et al, 2021
Example: COPII proteins on budding GUVs

Hutchings et al, 2021
Example: COPII proteins on budding GUVs

Hutchings et al, 2021
Example: HIV-1 trimer single particle
Example: Tomography for single particle initial model

- Classes used as templates for picking single particle micrographs
- Single particle now at 4 Å without anisotropy.

Jillian Chase and Alex Noble
eLife, 2018 and 2019
Example: HIV-1 Capsid-SP1 at 3.9/3.4/3.2/3.0 Å

- Krios + Super-res K2 + Gatan Energy Filter
- Fiducial tilt-series alignment
- 1.5 – 5 micron defocus
- Strip-based CTF correction
- ~750,000 sub-particles used
- TOM, AV3, Dynamo, and in-house scripts were used
- NovaCTF 3D CTF pushed it to 3.4 Å
- emClarity pushed to 3.2 Å
- Warp/M to 3.0 Å
Example: HIV-1 Capsid-SP1 at 3.9 Å

An atomic model of HIV-1 capsid-SP1 reveals structures regulating assembly and maturation
Warp/M Co-sub-tilt-series refinement

\[ S_i = \text{Projection}(\text{pose}_i) \cdot \text{Image}_i \]

\[ S_n = \text{Projection}(\text{pose}_n) \cdot \text{Image}_n \]

Multi-particle system, optimized simultaneously

\[ M = \sum_{s}^{N_{\text{spines}}} \sum_{p}^{N_{\text{templates}}} \sum_{t}^{N_{\text{frames}}} \text{Projection}(\text{pose}_{s,p,t} + \text{correction}_{s,p,t}) \cdot \text{Image}_{s,p,t} \]
Warp/M Co-sub-tilt-series refinement: apoferritin

Tegunov et al., 2021
Warp/M Co-sub-tilt-series refinement: *In-situ* 70S ribosome

Tegunov et al., 2021
Warp/M Co-sub-tilt-series refinement: Dynamo-Warp/M-Relion workflow

teamtomo.org
Refining tilt-series alignment by tracking just particles

Himes et al., 2017
BISECT: Higher-throughput parallel acquisition

Bouvette et al., 2021
BISECT: Higher-throughput parallel acquisition
Refining tilt-series alignment by tracking beads in 3D
Refining tilt-series alignment by tracking beads in 3D
Processing/Resolution limits

- Pixelsize (highest resolution = 2 x pixelsize = **Nyquist**)
- Isotropic motion (monitor your **drift** before full collection)
- Inherent specimen **flexibility**
- **Ice warping** in 3D during collection (doming)
- Beam-induced **motion of objects** of interest **in 3D** (particularly anisotropic)
Current/future directions in tomography
Future hardware improvements in the field:
3D cryo-CLEM
Hardware improvement – Rapid tilting

<table>
<thead>
<tr>
<th>Nominal magnification</th>
<th>Pixel size (Å)</th>
<th>Exposure time (s)</th>
<th>Total frames</th>
<th>Total time per tilt-series (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33kx</td>
<td>4.32</td>
<td>126</td>
<td>5040 or less</td>
<td>9.7</td>
</tr>
<tr>
<td>53kx</td>
<td>2.74</td>
<td>50</td>
<td>2000 or less</td>
<td>7.6</td>
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<tr>
<td>81kx</td>
<td>1.78</td>
<td>20</td>
<td>800</td>
<td>6.7</td>
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<tr>
<td>130kx</td>
<td>1.09</td>
<td>12</td>
<td>480</td>
<td>5.0</td>
</tr>
</tbody>
</table>

MOSTLY ALL cryotomography, MOST ALL the time

Chreifi et al., 2019
Hardware/software improvement

Pre-calibrated rapid tilting!

Fast-incremental single-exposure

Tilt series movie

Subtomogram average at subnanometer resolution

Collection

< 5 min per tilt series

Processing

several days

K3

x, y, z specimen shift compensation

Eisenstein et al., 2019
Software improvements in the field
Pre-calibrated Rapid tilting

Eisenstein et al. 2019
Post-processing improvement - *Denoising* Cryo-CARE (3D Noise2Noise):
Post-processing improvement - *Denoising*

**Cryo-CARE (3D Noise2Noise):**

Before

After!
Post-processing improvement - *Denoising*

Topaz (3D Noise2Noise):
Further progress:
- New/better modules
- Cross-talk between the modules
- Standardized IO

Unreleased and interesting:
- Bartaesgili et al. 2008
- Warp ~ Tegunov et al. (Bioarxiv)
- PyTOM workflow

~16 operations of various difficulty
~5 image interpolations

Do it some early time
- Dose Weighting
  - Motioncor2, Unblur, Relion
- Throw away bad projections
- Per tilt CTF determination
  - GCTf, CTFFind, Ctffplotter, TOMOCt
  - EmClarity
- Anisotropic mag correction
  - Motioncor2, Relion, PyTOM

Produced with input from
- Alex J. Noble (NYSCC)
Thank you!

Questions?

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National Resource for Automated Molecular Microscopy
Simons Electron Microscopy Center
New York Structural Biology Center