

Table S1. Comparison of features among major sub-tomogram averaging software packages

	emClarity [‡]	pyTOM ¹	Jsub-Tomo ²	Dynam ³	Eman2 ⁴	RELION ⁵	PEET ⁶	Protomo/i3 ⁷
GPU support	Yes	No	No	Yes	No	No	No	No
GUI	No	No		Yes		No	Yes	No
Template Matching	Yes	Yes	No	Manual	Manual	No	Manual	Yes
3D-CTF correction	3D-CTF WBP	No/Yes	No	No	Per-particle	Per-particle	No	No
Missing-wedge Representation	3D-Sampling Function	Binary Wedge	Binary Wedge	Binary Wedge	Fourier Intensity threshold	3D-Sampling Function	Binary Wedge	Binary Wedge (Sharp)
Tilt-series refinement using sub-volumes	Yes	No	No	No	No	No	No	No
“Gold-standard”	Yes FOM weighting	Yes FOM weighting	No	Yes	Yes Ad-Hoc weighting	Yes FOM weighting	Yes	No
Classification	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes

‡ This work

1. Hrabe, T. *et al.* PyTom : A python-based toolbox for localization of macromolecules in cryo-electron tomograms and subtomogram analysis. *J. Struct. Biol.* **178**, 177–188 (2012).
2. Maurer, U. E. *et al.* The structure of herpesvirus fusion glycoprotein B-bilayer complex reveals the protein-membrane and lateral protein-protein interaction. *Structure* **21**, 1396–1405 (2013).
3. Castaño-Díez, D., Kudryashev, M., Arheit, M. & Stahlberg, H. Dynamo: A flexible, user-friendly development tool for subtomogram averaging of cryo-EM data in high-performance computing environments. *J. Struct. Biol.* **178**, 139–151 (2012).
4. Galaz-Montoya, J. G., Flanagan, J., Schmid, M. F. & Ludtke, S. J. Single particle tomography in EMAN2. *J. Struct. Biol.* **190**, 279–290 (2015).
5. Bharat, T. A. M. & Scheres, S. H. W. Resolving macromolecular structures from electron cryo-tomography data using subtomogram averaging in RELION. *Nat. Protoc.* **11**, 2054–2065 (2016).
6. Heumann, J. M., Hoenger, A. & Mastronarde, D. N. Clustering and variance maps for cryo-electron tomography using wedge-masked differences. *J. Struct. Biol.* **175**, 288–299 (2011).
7. Winkler, H. 3D reconstruction and processing of volumetric data in cryo-electron tomography. *J. Struct. Biol.* **157**, 126–137 (2007).

Table S2. Run times for the yeast 80s tutorial data^a

Steps	Time	# of GPUs?
Average	1h 31m	2
Align	9h 45m	2
Tomo-CPR	2h 10m	2
3D-CTF	1h 16m	2
CTF update	0h 21m	2
Template Matching	0h 12m (1h 24m)	2
Classification	0h 8m	2
Total	15h 20m	

Footnote: a. Run on a Samsung 850-pro solid state scratch disk, requiring ~ 520 Gb total space. 2 x 20x Intel Xeon CPU E5-2650 v2 @ 2.3 GHz (only 12 cores used), 512 Gb of memory available, ~ 32 Gb used. 2x Nvidia Titan V GPUs.

Table S3. Alignment details for the ribosome data. The yeast 80s (EMPIAR-10045) and mammalian 80s (EMPIAR-10064) differ only in the size of the mask, and particle mass as noted in the bottom row.

Cycle	Binning	Pixel	Angular Search	Mask Type	Mask Radius (Å)	Particle Radius (Å)	Mass (MDa)		
template matching	5	10.85	[180, 15, 180, 15]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
0	4	8.68	[0, 0, 16, 4]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
1	4	8.68	[15, 5, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
2	4	8.68	[0, 0, 18, 2]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
3	3	6.51	[12, 4, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
4	3	6.51	[0, 0, 9, 1.5]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
5	3	6.51	[7.5, 2.5, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
6	2	4.34	[7.5, 2.5, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
7	2	4.34	[0, 0, 6, 1]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
8	2	4.34	[4.5, 1.5, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
9	1	2.17	[4.5, 1.5, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
10	1	2.17	[0, 0, 5, 0.5]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
11	1	2.17	[3.75, 1.25, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
12	1	2.17	[1.5, 0.75, 0, 0]	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
13	1	2.17	N/A	sphere	[180, 180, 180]	[160, 160, 160]	3.5		
							mammalian + 20	mammalian + 20	mammalian + 1

Table S4. Alignment details for the HIV-1 Gag data (EMPIAR-10164)

Cycle	Binning	Pixel	Angular Search	Mask Type	Mask Radius (Å)	Particle Radius (Å)	Mass (MDa)
template matching	7	7	[180, 9, 28, 7]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
0	6	6	[0, 0, 24, 3]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
1	6	6	[16, 4, 0, 0]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
2	6	6	[0, 0, 9, 1.5]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
3	5	5	[0, 0, 12, 1.5]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
4	5	5	[15, 3, 0, 0]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
5	5	5	[0, 0, 9, 1]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
6	4	4	[0, 0, 9, 1]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
7	4	4	[10, 2, 0, 0]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
8	4	4	[0, 0, 7.5, 0.75]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
9	3	3	[9, 1, 0, 0]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
10	3	3	[10, 2, 0, 0]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
11	3	3	[0, 0, 7.5, 0.75]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
12	2	2	[0, 0, 7.5, 0.75]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
13	2	2	[5, 1.25, 0, 0]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
14	2	2	[1.5, 0.75, 0.5, 0.5]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
15	2	2	[0, 0, 6, 0.5]	cylinder	[116, 116, 72]	[66, 66, 56]	0.5
16	1	1	[0, 0, 3, 0.3]	cylinder	[90, 90, 72, 72]	[66, 66, 56]	0.5
17	1	1	[4.5, 0.75, 0, 0]	cylinder	[90, 90, 72, 72]	[66, 66, 56]	0.5
18	1	1	[1.2, 0.4, 0, 0]	cylinder	[90, 90, 72, 72]	[66, 66, 56]	0.5