**Supplementary Information**



**Figure S1.** Comparison of resolution of per-frame reconstruction of various samples frozen under different freezing conditions. **A-C.** The comparison of per-frame reconstructions of datasets collected in pink region (red curves) and that collected in grid frozen at -183 ℃ in conventional conditions (blue curves) without intentionally moving the tweezers towards the center of grid. **D-E.** The comparison of per-frame reconstructions of datasets frozen at -110 ℃ (red curves) and frozen at -183 ℃ in conventional freezing conditions (blue curves).



**Figure S2.** The calibration curve and fitting formula of the thermocouple. The actual temperature was measured from the liquid nitrogen (-196 °C) and the solid liquid phase of pure water (0 °C), acetone (-95 °C), ethanol (-114 °C), diethyl ether (-116 °C) and ethane (-183 °C) at an atmospheric pressure, respectively. Six data points were well fitted to an exponential function (black).



**Figure S3**. Samples including apo-ferritin, GDH aldolase and β-galactosidase embedded in vitreous ice frozen at −110 °C.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Samples** | **T (℃)** | **Grid** | **Device****of****plunge** | **Electron microscope** | **M a** | **Binned pixel size****(Å/pixel)** | **Dose rate****(e-/Å2/s)** | **Total****dose****(e-/Å2)** | **BIS b (Y/N)** | **Energy filter****(eV)** | **Images number** | **Particles number** | **Resolution****(Å)** |
| apo-ferritin | -183 | NiTi filmon Au grid | Gatan CP3 | Titan Krios | 165000 | 0.82 | 12 | 60 | N | N | 287 | 68501 | 2.2 |
| GDH | -183 | carbon filmon Cu grid | Gatan CP3 | Titan Krios G3 | 165000 | 0.82 | 12 | 60 | Y | 20 | 58 | 6363 | 3.3 |
| VLP | -183 | NiTi filmon Au grid | Leica EMGP | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 54 | 1148 | 3.6 |
| apo-ferritin | -150 | NiTi filmon Au grid | Gatan CP3 | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 382 | 76573 | 2.4 |
| apo-ferritin | -110 | NiTi filmon Au grid | Gatan CP3 | Titan Krios G2 | 265000 | 0.515 | 25.1 | 60 | N | 20 | 1370 | 296695 | 1.9 |
| aldolase | -110 | NiTi filmon Au grid | Gatan CP3 | Titan Krios | 165000 | 0.82 | 12 | 60 | N | N | 313 | 46253 | 3.1 |
| aldolase | -110 | carbon filmon Cu grid | Gatan CP3 | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 592 | 158416 | 4.0 |
| GDH | -110 | carbon filmon Cu grid | Gatan CP3 | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 285 | 49665 | 3.0 |
| apo-ferritin | standard | NiTi filmon Au grid | VitroBot | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 560 | 103770 | 2.1 |
| GDH | standard | carbon filmon Cu grid | Gatan CP3 | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 790 | 83207 | 2.8 |
| VLP | standard | carbon filmon Cu grid | Leica EMGP | Titan Krios G2 | 130000 | 1.04 | 9.2 | 60 | N | 20 | 587 | 18882 | 3.0 |
| aldolase | standard | NiTi filmon Au grid | Gatan CP3 | Titan Krios G2 | 165000 | 0.82 | 12 | 60 | N | 20 | 295 | 55989 | 4.0 |

**Table S1.** Information on cryo-EM specimen preparation, data collection and structure determination for all samples.

a Magnification.

b BIS is beam-image shift data collection ([Wu, Huang, Cheng, Zhu, & Zhang, 2019](#_ENREF_1)).

**Table S2.** The temperature calibration of EMGP and CP3 using the temperature thermocouple.

|  |  |
| --- | --- |
| **EMGP** | **CP3** |
| **Readings of EMGP/°C** | **Actual temperatures /°C** | **The differences between EMGP and actual temperature/°C** | **Readings of CP3/°C** | **Actual temperatures /°C** | **The differences between CP3 and actual temperature/°C** |
| -180 | -181 | -1 | -180 | -186 | 6 |
| -170 | -171 | -1 | -170 | -176 | 6 |
| -160 | -160 | 0 | -160 | -166 | 6  |
| -150 | -150 | 0 | -150 | -156 | 6 |
| -140 | -140 | 0 | -140 | -146 | 6 |
| -130 | -130 | 0 | -130 | -136 | 6 |
| -120 | -120 | 0 | -120 | -125 | 5 |
| -110 | -110 | 0 | -110 | -115 | 5 |
| -100 | -100 | 0 | -100 | -105 | 5 |
| -90 | -90 | 0 | -90 | -95 | 5 |
| -80 | -80 | 0 | -80 | -85 | 5 |

Wu, C., Huang, X., Cheng, J., Zhu, D., & Zhang, X. (2019). High-quality, high-throughput cryo-electron microscopy data collection via beam tilt and astigmatism-free beam-image shift. *Journal of structural biology*.