Protocol for method of Nijsse, Cruz, and Melnikov, 2024.

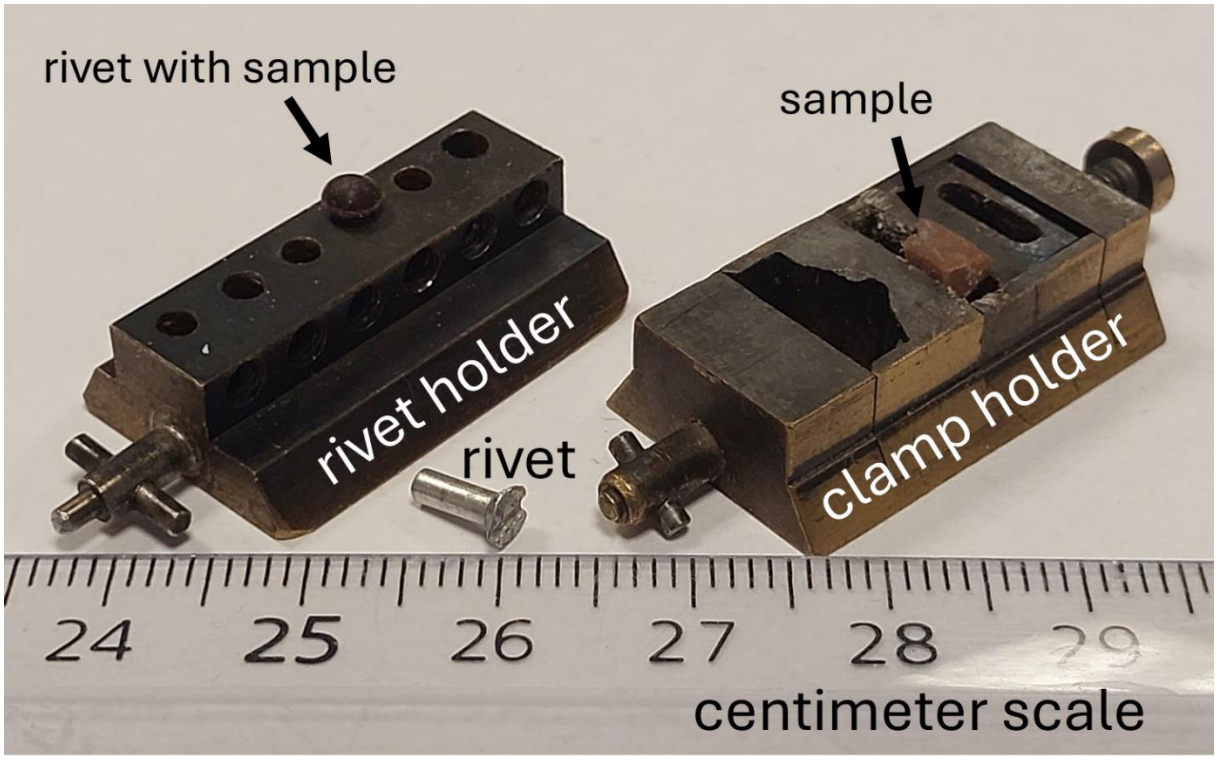
Microstructure of confectionery masses revealed by cryo-planing

1. Sampling and fixation

   Choose either option 'a' (includes melting of the chocolate) or 'b' (keeps solid samples in the solid state)

   a. The chocolate is melted, and a droplet is put on top of a rivet (see figure 1). The chocolate is allowed to solidify*, and the rivet is plunge-frozen in liquid nitrogen. The sample is mounted onto a rivet holder (see figure).

   b. A piece of chocolate of several millimeters length, width, and height, is cut out using a razor blade, by chipping off thin slices until a piece of the desired dimensions remains. This piece is placed in a clamp-holder (see figure)



   Both the rivet holder and the clamp holder fit into the ultramicrotome as well as into the cryo-SEM, as well as into the CLSM. By using a single holder, there is no need for re-mounting of the sample during the subsequent steps, and this also ensures a perpendicular view of the polished block surface in both SEM and CLSM. These custom-built sample holders were acquired from Unilever R&D. Depending on the configuration of a specific microtome and microscopes a holder with similar functionality should be made/used.

Now the clamp-holder with the clamped sample is plunge-frozen in liquid nitrogen.

2. Cryo-planing of the sample
    a. A cryo-ultramicrotome is used (Leica Ultracut UCT EM-FCS). The sections are discarded, a polished block face remains.
    b. Temperature setting: -110°C (area, knife, and sample)
    c. An antistatic gun is used to remove the sections from the planed surface
    d. First sections are made with a glass knife at 200nm per section and speed of 60 mm/s. When reaching a desired plane, the thickness and speed are reduced to 50 nm and 20 mm/s.
    e. Final sections are made with a diamond knife (Diatome, Histo-Cryo-Dry-8mm), starting at 50 nm and 20 mm/s and reducing the speed to 2 mm/s at same slice thickness.
    f. Cryoplaning is finished once a flat, mirror-like surface has been created.
    g. The sample is quickly transferred from air to vacuum (so not necessarily through liquid nitrogen), into the cryo-preparation-chamber (Gatan Alto 2500) of the SEM (Jeol 6490LA).
    h. In the high vacuum of the cryo-preparation chamber of the SEM, the sample is sublimated at -80°C, until all frost has disappeared
3. Analysis of the sample in cryo-SEM
    a. The sample is imaged in the SEM with the following settings:
        i. -40°C, 40Pa, 15kV, with a wide objective lens aperture and high spotsize settings (here 60).
        ii. imaging with a Backscattered Electron Detector (Jeol MP-44160BEIW, having a Si P-N type semiconductor detector), set to the compositional contrast (BEC) contrast. Brightness is adjusted such that the fat phase appears dark grey, and Contrast is adjusted such to obtain a grey level differentiation between the different ingredient particles.
        iii. Elemental analysis is done with the in-built Jeol-EDS-system, by spot analysis.
4. Analysis of the sample with CLSM
    a. The clamp-holder containing the sample is taken out of the SEM, and kept for at least 15 minutes in the vacuum of the cryo-transfer-device, to allow the sample to reach room temperature. Then the transfer-device is opened and the clamp holder is positioned in the CLSM microscope (Leica SP5 inverted setup).
    b. The sample is analyzed by detecting autofluorescence (excitation wavelengths: 405nm and 488nm; emission channel bands: blue 413-467nm, green 517-577nm, red 661-788nm).
    c. A dry lens (10x) is used for low magnifications, and an oil lens (63x) is used fo high magnifications. In the latter case, the immersion oil is applied directly

between the lens and the polished sample surface. After imaging, the lens is cleaned with two rinses of immersion oil.

d. Imaging is typically done at 1024x1024 pixels and scanspeed of 100 Hz, either using a single focal-slice or a projection of several consecutive focal slices, depending on the research question.

*The sample could be cryofixed right from a liquid state (originally liquid or melted), but in that case only the SEM analysis can be done, but not the here described additional CLSM method.