Appendix III: Microscopic Identification

Microscopic identification is a method using a microscope to identify the characters of structural features, cells and ergastic substances in section, powder, disintegrated tissue or surface slides of CMM samples. Select a representative CMM sample and conduct the examination in accordance with the methods described below.

(1) **Preparation of cross and/or longitudinal sections slides** – Select a representative sample for examination. After softening, cut the material with a razor blade or a sliding microtome to a thickness of 10-20 μm. Examine the sample under a microscope after treated with glycerol-acetic acid TS, chloral hydrate TS or other suitable TS. Embed the material in hard paraffin for cutting, when necessary.

(2) **Preparation of powder slides** – Spread a small quantity of the powder on a slide, treated with glycerol-acetic acid TS, chloral hydrate TS, or other suitable TS and conduct the examination. When necessary, add heat to increase transparency in the procedure.

(3) **Preparation of surface slides** – After moistening and softening, cut the sample apart or tear out the epidermis, add suitable TS and conduct the examination.

(4) **Maceration and preparation of slides** – Cut or slice the sample into small pieces of about 2 mm in thickness for maceration. Depending on the nature of the material, one of the following three methods is used –

(a) **Potassium hydroxide method** – Place the sample in a test tube, and add an adequate quantity of aqueous potassium hydroxide solution (5%, v/v), then heat until the residue can be easily separated when pressed with a glass rod. Decant the alkaline solution and wash the residue with water, transfer a small amount of macerated material onto a slide and tease it out with a needle. Mount in dilute glycerine and examine under a microscope.

(b) **Chromic-nitric acid method** – Place the sample in a test tube and add an adequate quantity of chromic-nitric acid TS, then let stand until the material can be easily separated when pressing with a glass rod. Decant the acidic solution, wash the residue with water, and prepare the slide as directed above in (4)(a).

(c) **Potassium chlorate method** – Place the sample in a test tube, add dilute nitric acid (50%, v/v) and a small quantity of potassium chlorate; warm gently until the effervescence subsides, then add small amount of potassium chlorate to maintain a slight effervescence. When the tissue shows a tendency to disintegrate, break the material with a glass rod. Decant the acidic solution, wash the macerated material with water and prepare the slide as directed above in (4)(a).

For CMM samples with only a few or scattered woody tissues or with parenchyma tissues, use potassium hydroxide method. Whereas, for hard material mainly with woody tissues or woody tissues grouped into bundles, use chromic-nitric acid or potassium chlorate method.

(5) **Measurements of sizes of cell and its contents** – Under a microscope, use an ocular micrometer to measure the sizes of cell and cell contents of specimens. Place the ocular micrometer scale in the eyepiece of the microscope, then calibrate with a stage micrometer. For the calibration, turn the eyepiece and move the stage micrometer to make the divisions on the two scales parallel and their left "O" lines coincide, then look for another line which coincides to the right.

The value (in micrometer) of one ocular micrometer division can be calculated on the basis of divisions of the two micrometer scales between the coinciding lines.

To measure the object, multiply the number of object-measuring divisions of ocular micrometer by the value (in micrometer) of each division. In general, it is carried out under a high power objective, but a low power objective would be more convenient to measure the lengths of longer fibres and non-glandular hairs, etc.

Record the maximal and minimal values (in micrometer), values slightly higher or lower than those specified in the individual monograph are acceptable.

(6) **Histochemical detection of cell walls** –

(a) **Lignified cell wall** – Add 1-2 drops of phloroglucinol TS to the specimen on the slide, allow to stand for a moment, then add 1 drop of hydrochloric acid. Lignified cell walls are stained red or purplish-red according to the extent of lignification.

(b) **Suberized or cuticular cell wall** – Add 1-2 drops of Sudan III TS to the specimen on the slide, allow it to stand for a few minutes or warm gently. Suberized or cuticular cell walls are stained orange-red or red.

(c) **Cellulose cell wall** – Add 1-2 drops of zinc chloride-iodine TS and allow to stand for few minutes; alternatively, add 1-2 drops of iodine TS, allow to stand for a while, and then add dilute sulphuric acid (66%, v/v). Cellulose cell walls are stained blue or purple.

(d) **Siliceous cell wall** – Add 1-2 drops of sulphuric acid, no change is observed.

(7) **Histochemical detection of cell contents** –

(a) **Starch** – (i) Add iodine TS, a blue or purple colour is observed; (ii) Mount in glycerol-acetic acid on slide, examine the slide under polarized microscope. Non-gelatinized starch granules shall show crosses under polarized light, which disappears upon gelatinization of the starch.

(b) **Aleurone** – (i) Add iodine TS, a brown or yellowish-brown colour is observed; (ii) Add mercuric nitrate TS, a brick red colour is observed. If the material is oily, render it fat-free by immersing in and washing with ether or petroleum ether before carrying out the test.

(c) **Fatty oil, volatile oil or resin** – (i) Add Sudan III TS, an orange-red, red or purplish-red colour is observed; and (ii) Irrigate the material with ethanol (90%), volatile oils are dissolved in the solvent, while fatty oils are insoluble (except castor oil and croton oil).

(d) **Inulin** – Add α-naphthol in ethanol (10%, w/v) and then add sulphuric acid, the crystals of inulin turn purplish-red and dissolve rapidly.

(e) **Mucilage** – Add ruthenium red TS, a red colour is observed.

(f) **Calcium oxalate crystals** – (i) Insoluble in dilute acetic acid (6%, v/v), soluble in dilute hydrochloric acid (9.5-10.5%, v/v) without effervescence; (ii) Dissolve gradually in dilute sulphuric acid (50%, v/v), needle crystals of calcium sulphate appear after stand for a moment.

(g) **Calcium carbonate (stalactile)** – Soluble in dilute hydrochloric acid (9.5-10.5%, v/v) with effervescence.

(h) **Silica** – Insoluble in sulphuric acid.

(8) **Preparation of test solutions (TS) for microscopic analysis** –

(a) **Chloral hydrate TS** – Dissolve 50.0 g chloral hydrate in a mixture of 15 mL of water and 10 mL of glycerine.

(b) **Cuoxam TS** – Add a sufficient amount of water to 0.5 g of copper carbonate and grind in a mortar, then add 10 mL of strong ammonia solution to dissolve.

(c) **Ferric chloride TS** – Dissolve 1.0 g of ferric chloride in water and make up to 100 mL.

(d) **Fuchsin glycerine gelatin** – Dissolve 1.0 g of animal gelatin in 6 mL of water, then add 7 mL of glycerine and heat with gentle stirring until well mixed; after filter through a piece of gauze into a Petri dish, add sufficient amount of basic fuchsin solution (dissolve 0.1 g of basic fuchsin in 600 mL of absolute ethanol and 80 mL of camphor oil), then mix well and allow to solidify.

(e) **Glycerol-acetic acid TS** – Mix well equal volumes of glycerine, glacial acetic acid and water.

(f) **Iodine TS** – Use 0.1 M iodine solution directly.

(g) **Mercuric nitrate TS** – Add 3 mL of fuming nitric acid to 4.5 g of mercury, when the reaction is completed, dilute with an equal volume of water. Preserved in an amber-coloured glass bottle with a glass stopper and protected from light.

(h) **α-Naphthol TS** – To 10.5 mL of a solution of α-naphthol in ethanol (15%, w/v), gently add 6.5 mL of sulphuric acid and mix well, then add 40.5 mL of ethanol and 4 mL of water, mix well.

(i) **Phloroglucinol TS** – Dissolve 1.0 g of phloroglucinol in 100 mL of ethanol (90%) and then filter. Preserved in an amber-coloured glass bottle and protected from light.

(j) **Ruthenium red TS** – Add a sufficient quantity of ruthenium red to 1-2 mL of aqueous sodium acetate solution (10%, w/v) to make a wine red colour. Freshly prepare the solution.

(k) **Sudan III TS** – Dissolve 0.01 g of Sudan III in 5 mL of ethanol (90%), then add 5 mL of glycerine and mix well. Preserved in an amber-coloured glass bottle, use within 2 months.

(l) **Tissue-disintegrating solution (chromic-nitric acid TS)** – (i) Add 10 mL of nitric acid to 100 mL of water and mix well; and (ii) Dissolve 10 g of chromic trioxide in 100 mL of water. Mix equal volumes of the above solutions (i) and (ii) prior to use.

(m) **Zinc chloride-iodine TS** – Dissolve 8.0 g of potassium iodide in 8.5 mL of water and add 2.5 g of anhydrous zinc chloride, the mixture is then saturated with sufficient quantity of iodine. Preserved in an amber-coloured glass bottle with a glass stopper.