

Supplementary Information

Title: Nucleus-specific X-ray stain for 3D virtual histology

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Supplementary Materials:

Staining Protocol according to histological hematoxylin-based procedures:

(a) Hematoxylin according to Mayer

The mouse organ was removed post mortem and immediately placed in a 50-ml Falcon Centrifuge Tube (neoLab), which was filled with a fixative solution containing 15ml of 4% (v/v) formaldehyde solution (FA, derived from a 37% acid free FA solution stabilized with ca. 10% methanol from Carl Roth; further dilution with DPBS without calcium and magnesium). The sample was refrigerated for 24-72 hours and then washed with phosphate saline buffer solution for 1hour (DPBS without calcium and magnesium). The mouse organ was placed in Mayer's staining solution (Morphisto). The soft-tissue sample was stained with 6ml of Mayer's staining solution for 72 hours (the soft-tissue sample was moving freely within the sample container). During the incubation time the soft-tissue sample was kept in the dark and on a horizontal shaking plate allowing for a smooth rocking of 60rpm. After staining the soft-tissue sample was carefully removed from the sample container and excess of staining agent was softly patted with a cellulose tissue paper. The soft-tissue sample was washed with 6ml tap water (washing solution was changed every hour for the first 5 hours) and kept overnight in the washing solution. During the incubation time the soft-tissue sample was kept in the dark and on a horizontal shaking plate allowing for a smooth rocking of 60rpm. The soft-tissue sample was stored in an Eppendorf tube above an ethanol vapor phase (the Eppendorf tube contained a few drops of 70% (v/v) ethanol at the bottom of the tube).

1 **(b) Hematoxylin according to Weigert**

2 Organ preparation was performed as described in a). The mouse organ was placed in
3 Weigert's staining solution (Working solution A and B were mixed in the ratio of 1/1 prior
4 to use; Morphisto). The soft-tissue sample was stained with 6ml of Weigert's staining
5 solution for 72 hours (the soft-tissue sample was moving freely within the sample
6 container). During the incubation time the soft-tissue sample was kept in the dark and on
7 a horizontal shaking plate allowing for a smooth rocking of 60rpm. After staining the
8 soft-tissue sample was treated as described in a).

9 **(c) Hematoxylin according to Weigert applying the stepwise staining procedure**

10 Organ preparation was performed as described in a). The mouse organ was placed in
11 Weigert's staining solution (B) (Morphisto). The soft-tissue sample was stained with 3ml
12 of Weigert's staining solution (B) for 72 hours (the soft-tissue sample was moving freely
13 within the sample container). During the incubation time the soft-tissue sample was kept
14 in the dark and on a horizontal shaking plate allowing for a smooth rocking of 60rpm. After
15 the first staining step, the soft-tissue sample was carefully removed and placed for a brief
16 time period in an Eppendorf tube above an ethanol vapor phase (Eppendorf tube
17 contained a few drops of 70% (v/v) ethanol at the bottom of the tube). Addition of 3ml of
18 Weigert's staining solution (A) (Morphisto) to the sample container was performed,
19 followed by thorough stirring of the reaction mixture. Immediate color change to purple
20 upon addition of staining solution (A) was observed. The soft-tissue sample was placed
21 back into the sample container and incubated for further 72 hours. During the incubation
22 time the soft-tissue sample was kept in the dark and on a horizontal shaking plate allowing

- 1 for a smooth rocking of 60rpm. After staining the soft-tissue sample was treated as
- 2 described in a).

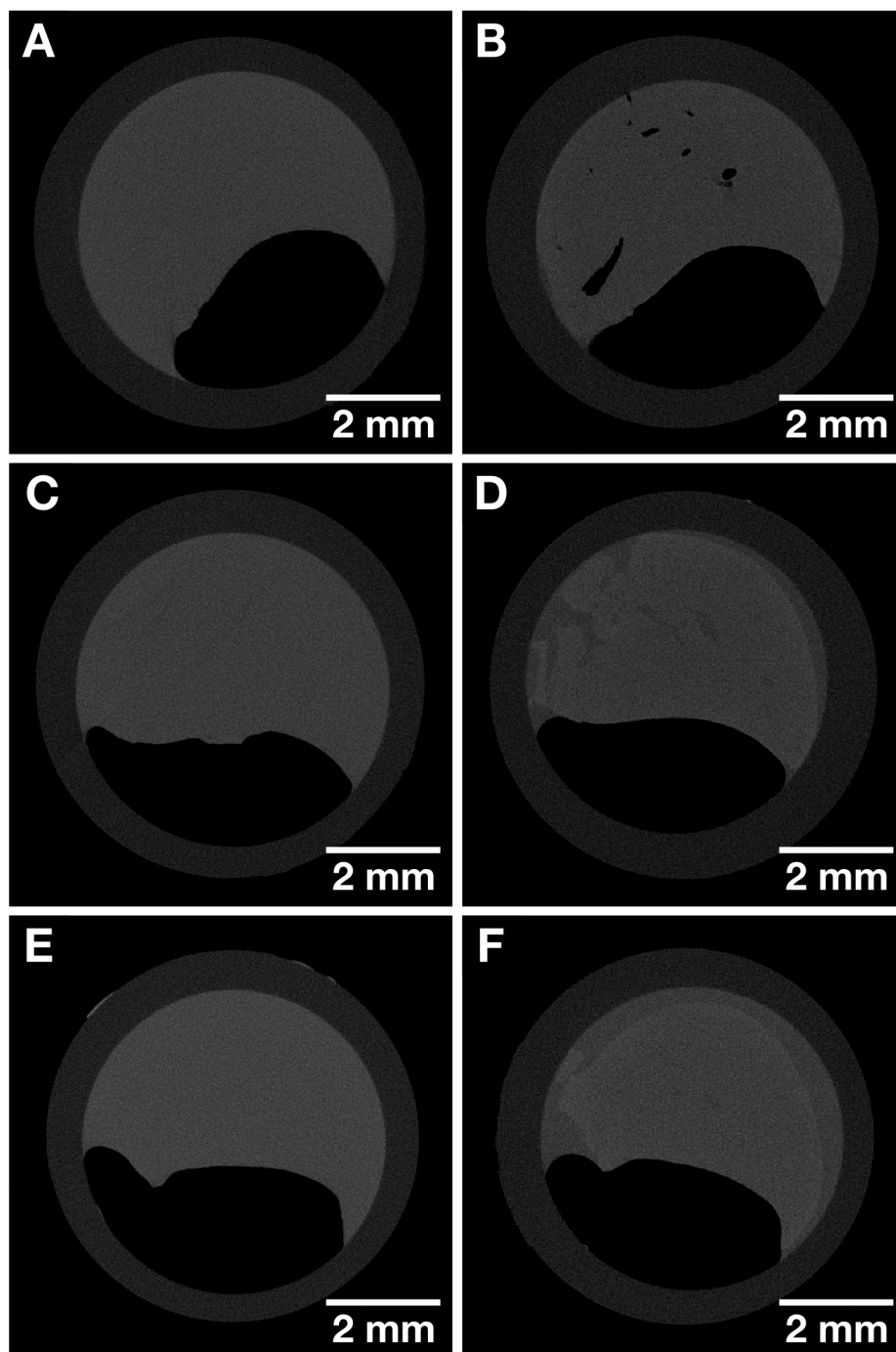


Figure S1. CT slices (xy plane) of the same whole mouse liver lobule before and after staining following standard histological staining protocols. The Xradia Versa 500 microCT was used to acquire all data sets under identical acquisition parameters. The voxel size in all data sets is around 14µm. **(A,B)** Overview images of an **(A)** unstained and **(B)** the very same stained mouse liver lobule with hematoxylin according to Mayer (15). **(C,D)** Overview images of an **(C)** unstained and **(D)** the very same stained mouse liver lobule with hematoxylin according to Weigert (16). **(E,F)** Overview images of an **(E)** unstained and **(F)** the very same stained mouse liver lobule with hematoxylin according to Weigert following the stepwise approach of the developed hematein-based staining procedure.

Movie S1: Animation showing 3D distribution of cell nuclei within the VOI. For 3D analysis the VOI has been compartmentalized in eight sub-cubes, whereby each sub-cube is visualized by dotted lines and numbered accordingly. Sub-cube one is highlighted in green for clarity.

Table S1: Parameters and conditions tested for the development and optimization of the X-ray suitable hematein staining protocol.

Parameters tested	Conditions tested
fixative	1% FA ¹ , 4% FA ¹ ; mixture: 1 % FA ¹ ; 2.5 % GA ²
pH	with / without glacial acetic acid
concentration of Pb(OAc) ₂ solution	333 mM, 666 mM
incubation time	6 h – 1 week (for individual steps each)
staining procedure	1 step protocol: working solutions A³ and B⁴ were freshly mixed prior to use 2 step protocol: i) 1st working solution A³ and 2nd addition of working solution B⁴ into the same sample container ii) 1st working solution B⁴ and 2nd addition of working solution A³ into the same sample container iii) 1st working solution A³ and 2nd working solution B⁴ (new sample container) iv) 1st working solution B⁴ and 2nd working solution A³ (new sample container)

¹FA = formaldehyde; ²GA = glutaraldehyde; ³working solution A: hematoxylin solution in absolute ethanol; ⁴working solution B: lead(II) acetate trihydrate solution in dist. Water.