

Phenotypic characterization of liver tissue heterogeneity through a next-generation 3D single-cell atlas

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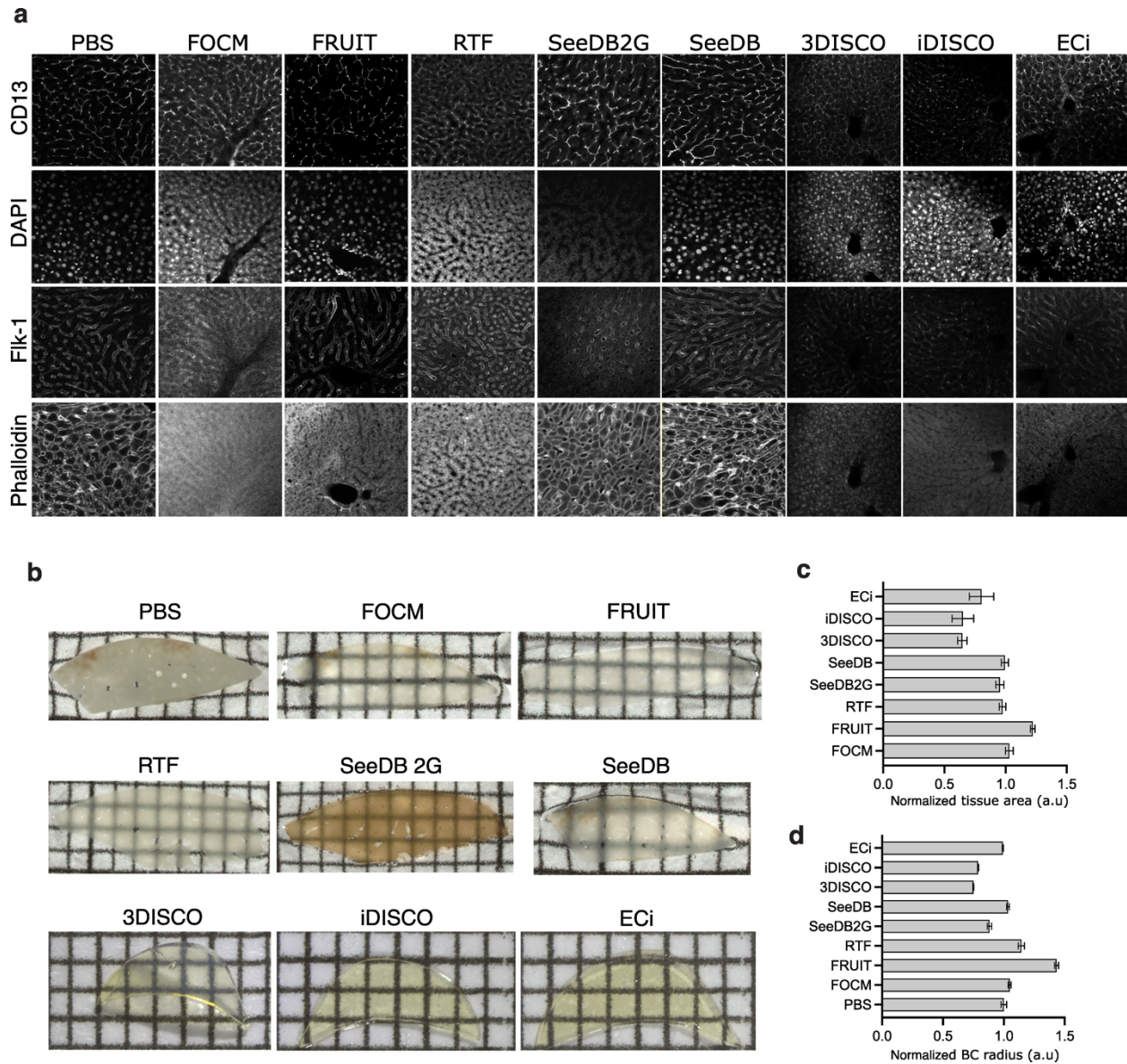
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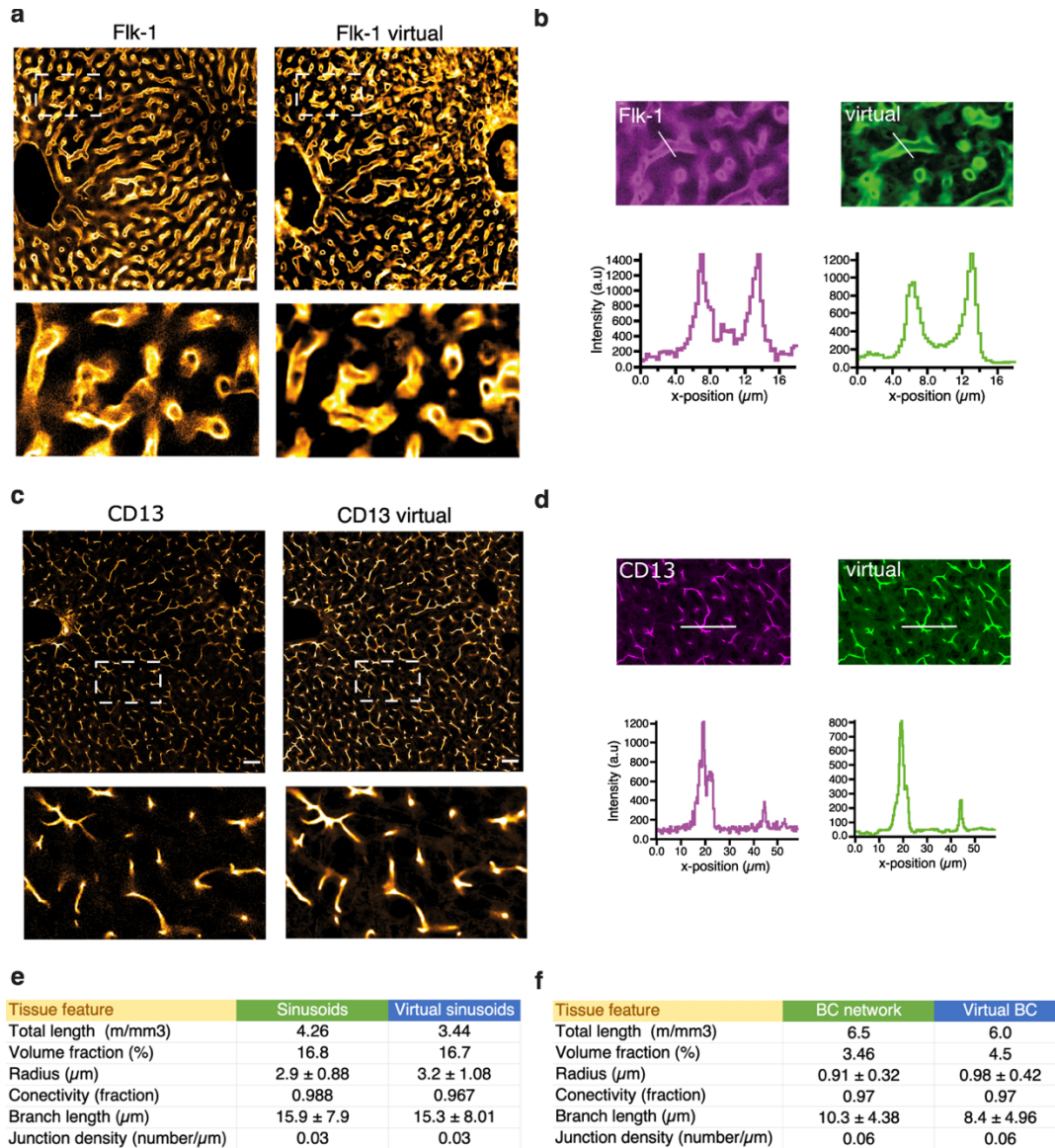
Supp movie 1: 3D single-cell morphometric atlas of liver tissue architecture.

Central vein (light blue), portal vein (orange), sinusoids (magenta), bile canaliculus (green), nuclei (random colours), hepatocytes (random colours), HSCs (random colours) and KCs (random colours).



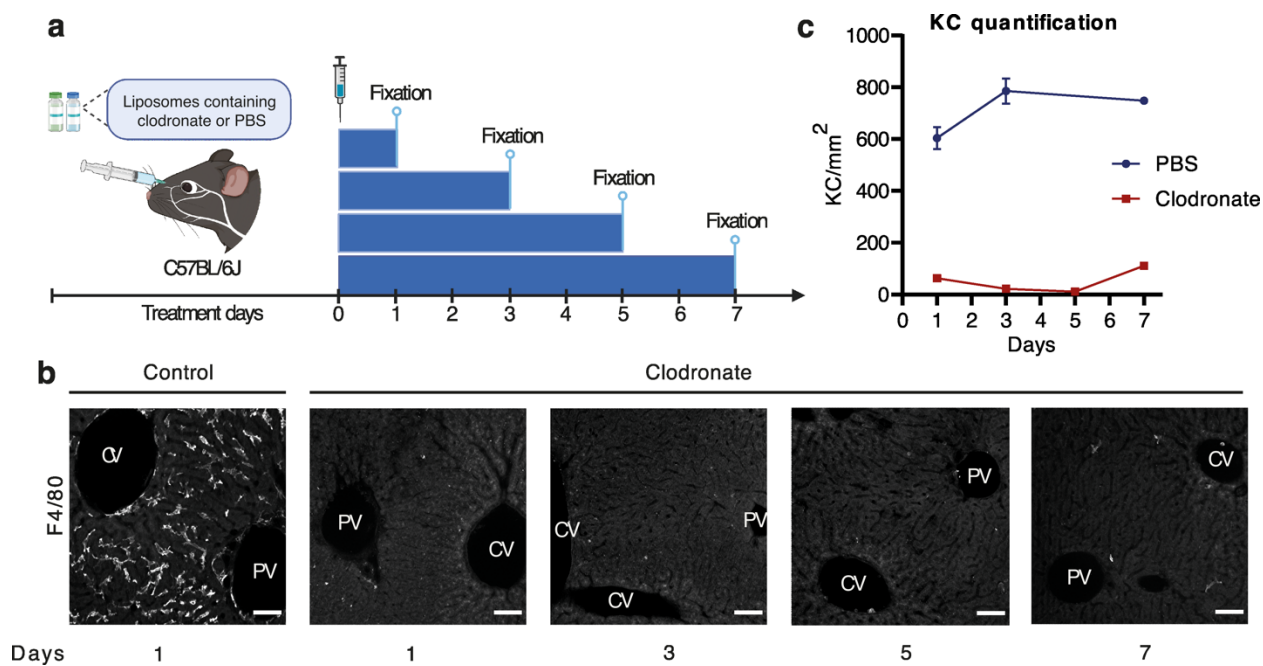
Supp Fig. 1: Comparison of different optical clearing methods.

(a) 100 μ m liver slices were stained with antibodies against CD13 and Flk-1, and the dyes phalloidin and DAPI. Different optical clearings methods were applied and the compatibility with the different markers was qualitatively evaluated. (b) Liver slices before and after the clearing are shown. (c) The outline of the liver slices was drawn in Fiji, the area was calculated and used as a readout of macroscopic tissue deformation. (d) BC was reconstructed with Motion Tracking and the radius was measured. The data were normalized to the PBS condition. The analysis was performed from three adult mice. Quantification represented by mean $\pm$ s.e.m.



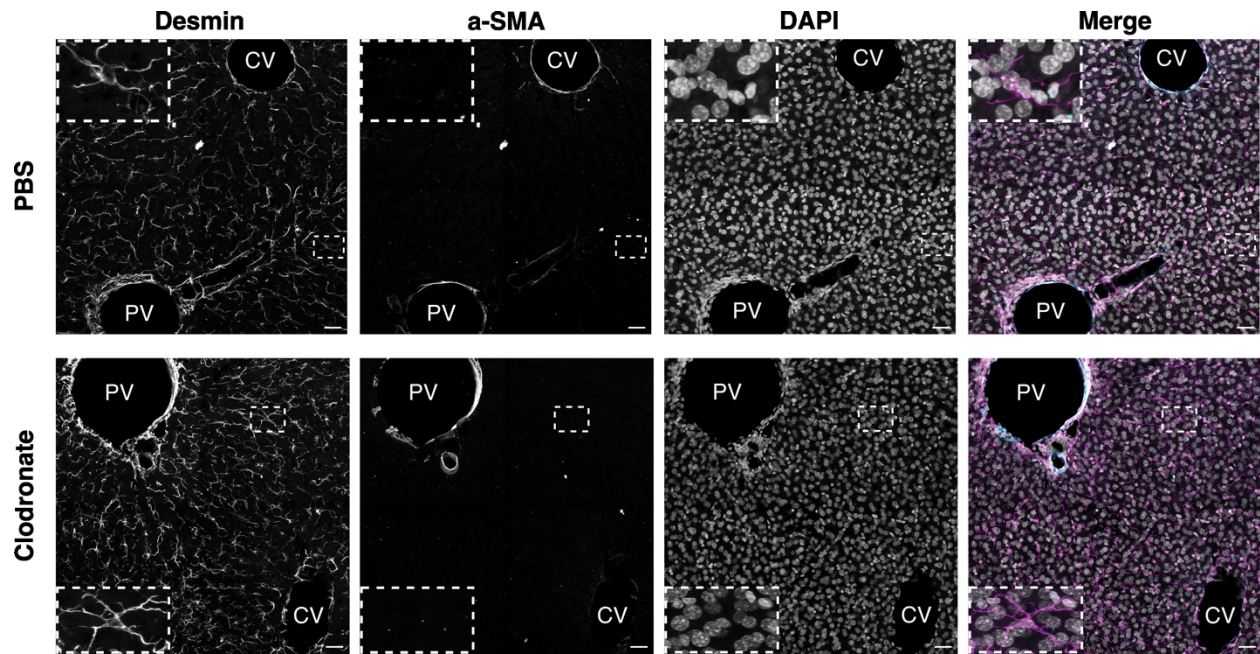
Supp Fig. 2: Artificial neural networks can create images that resemble real staining.

(a) Representative images of the sinusoids from fixed mouse liver tissue. On the left, liver section stained with Flk-1. On the right, virtual image created with the 3D CNN-based toolbox from the phalloidin staining. Inset showing a magnification of the region highlighted on the upper image. (b) Intensity profiles along the lines drawn on two examples of real marker (magenta) versus virtual (green). (c) Representative images of the BC from fixed mouse liver tissue. On the left, liver section stained with CD13. On the right, virtual image created with the 3D CNN-based toolbox from the phalloidin staining. Inset showing a magnification of the region highlighted on the upper image. (d) Intensity profiles along the lines drawn on two examples of real marker (magenta) versus virtual (green). (e-f) Real and virtual images were 3D reconstructed and some morphometric properties were quantified. The training of the models involved approximately 36000 (for BC) and 25000 (for sinusoids) non-overlapping patches derived from six Z-stacks obtained from postnatal day 1, 16, and adult mice.



Supp Fig. 3: Standardization of Kupffer cells depletion with clodronate liposomes.

(a) Scheme summarizing the depletion experiment. A single clodronate injection was performed and liver samples were obtained after 1, 3, 5 and 7 days. (b) 100 μ m thick liver sections were stained with anti-F4/80 antibody. (c) Number of KCs were quantified in Fiji using the images shown in (b) and were compared to control mice injected with PBS. PBS = 3 samples, Clodronate = 3 samples. Quantification represented by mean $\pm$ s.e.m.



Supp Fig. 4: HSCs do not get activated upon long-term KCs depletion.

Liver sections from mice treated with PBS and clodronate were stained with desmin, α -SMA and DAPI. The images represent the maximum projection of a 30 μ m z-stack covering an entire CV-PV axis. PBS = 3 samples, Clodronate = 3 samples. Scale bar 30 μ m.

Supp table 01: Morphometric analysis of BC and sinusoidal networks from early postnatal development to adulthood.

Bile canaliculi	Age	P1	P16	Adult
	Total length (m/mm³)	4.46 ± 0.5	6.17 ± 0.5	4.62 ± 0.3
	Volume fraction (%)	6 ± 0.47	5.4 ± 0.84	4.4 ± 0.59
	Radius 2D (μm)	1.4 ± 0.08	1.1 ± 0.04	1.1 ± 0.1
	Connectivity	0.84 ± 0.033	0.85 ± 0.023	0.88 ± 0.008
	Branch length (μm)	8 ± 1.4	8.1 ± 0.9	8.3 ± 0.5
	Junction density (number/μm)	0.06 ± 0.001	0.06 ± 0.001	0.06 ± 0.002

Sinusoids	Age	P1	P16	Adult
	Total length (m/mm³)	5.38 ± 0.4	6.65 ± 0.4	4.75 ± 0.3
	Volume fraction (%)	13.9 ± 0.7	27.1 ± 0.88	25.2 ± 4.64
	Radius 2D (μm)	2.5 ± 0.04	3.2 ± 0.1	3.5 ± 0.26
	Connectivity	0.93 ± 0.033	0.99 ± 9.5e-5	0.99 ± 0.003
	Branch length (μm)	12.8 ± 0.1	12.8 ± 0.2	14.9 ± 0.6
	Junction density (number/μm)	0.03 ± 0.001	0.04 ± 9.4e-5	0.04 ± 0.001

Supp table 02: Morphometric characterization of hepatocytes from early postnatal development to adulthood.

HEPATOCYTES (GENERAL)	Age	P1	P16	Adult
	Total number/mm ³	474336 ± 35903	595904 ± 33765	235756 ± 31938
	Volume fraction (%)	58.5 ± 1.4	59.6 ± 0.56	56.7 ± 2.22
	Cell volume (μm ³)	2152 ± 160	1731 ± 94	4471 ± 525
	Mono-nuclear (%)	75.8 ± 4.1	79.3 ± 7.6	63.1 ± 7.5
	Bi-nuclear (%)	17.3 ± 2.1	16.9 ± 4.7	34.5 ± 7.1
	Nuclei volume (μm ³)	344 ± 47	342 ± 40	480 ± 71
	Nuclei elongation	0.83 ± 0.050	0.52 ± 0.061	0.4 ± 0.057

HEPATOCYTES (BY PLOIDY)	Ploidy	1 x 2n			1 x 4n			1 x 8n		
	Age	P1	P16	Adult	P1	P16	Adult	P1	P16	Adult
	Percentage of total hepatocytes (%)	58.9 ± 2.7	52.4 ± 1.2	27.4 ± 0.8	15.5 ± 4.3	24.9 ± 5.5	27.9 ± 4.4	1.4 ± 1.2	1.6 ± 0.8	7.1 ± 3.7
	Cell volume (μm ³)	1412 ± 82	1247 ± 128	2175 ± 291	1821 ± 329	1612 ± 107	3756 ± 410	2801 ± 286	3250 ± 483	5949 ± 551
	Elongation	0.78 ± 0.006	0.59 ± 0.025	0.65 ± 0.015	0.94 ± 0.022	0.59 ± 0.026	0.57 ± 0.054	1.06 ± 0.141	0.75 ± 0.065	0.52 ± 0.050
	Apical surface (%)	15.4 ± 0.5	18.2 ± 0.8	17.8 ± 3.3	15.8 ± 0.9	18.9 ± 0.5	19.8 ± 3.0	16.9 ± 3	13.2 ± 2.5	22.7 ± 2.3
	Basal surface (%)	23.1 ± 1.2	39.1 ± 2.7	32.5 ± 2.7	23.3 ± 2.5	40.6 ± 3.1	39.6 ± 4	23.8 ± 2.9	50.2 ± 7.1	42.8 ± 3.8
	Lateral surface (%)	61.5 ± 1.4	42.7 ± 3.6	49.7 ± 5.9	60.9 ± 3.4	40.5 ± 3.6	40.6 ± 7	59.3 ± 5.8	36.6 ± 6.7	34.5 ± 6.1
	Number of neighbours	9 ± 0.24	8 ± 0.48	8 ± 0.33	10 ± 0.69	8 ± 0.57	9 ± 0.57	11 ± 1.4	11 ± 0.33	11 ± 0.93

Ploidy	2 x 2n			2 x 4n			2 x 8n		
Age	P1	P16	Adult	P1	P16	Adult	P1	P16	Adult
Percentage of total hepatocytes (%)	13.6 ± 2.7	12.6 ± 5.1	15.0 ± 2.8	0.5 ± 0.2	1.6 ± 0.6	15.7 ± 4.5	0 ± 0	0.1 ± 0.7	1.0 ± 0.5
Cell volume (μm ³)	3352 ± 178	2586 ± 308	4474 ± 529	3533 ± 782	3220 ± 349	7279 ± 596	0 ± 0	1482 ± 1482	9792 ± 603
Elongation	1.07 ± 0.038	0.93 ± 0.030	0.65 ± 0.050	1.06 ± 0.112	1.12 ± 0.038	0.67 ± 0.058	0 ± 0	0.38 ± 0.377	0.84 ± 0.144
Apical surface (%)	19.5 ± 1.7	19.7 ± 1.8	23.3 ± 3	19.3 ± 1.2	21.3 ± 1.3	22.3 ± 2.1	0 ± 0	9 ± 9	17.4 ± 3.7
Basal surface (%)	25.6 ± 0.9	41.1 ± 2.2	38.0 ± 3.6	21.9 ± 8.6	43.5 ± 2.9	44.7 ± 4.7	0 ± 0	11.5 ± 11.5	48.6 ± 7.4
Lateral surface (%)	54.9 ± 2.6	39.2 ± 3.9	38.7 ± 6.3	58.7 ± 7.5	35.3 ± 4.2	33 ± 6.6	0 ± 0	12.9 ± 12.9	34.0 ± 10.3
Number of neighbours	13 ± 0.02	10 ± 0.45	11 ± 0.68	13 ± 1.22	12 ± 0.97	12 ± 0.49	0 ± 0	4 ± 4	13 ± 0.5

Supp table 03: Morphometric characterization of non-parenchymal cells from early postnatal development to adulthood.

HSC	Age	P1	P16	Adult
	Total number/mm ³	61449 ± 11523	63983 ± 4805	44217 ± 7397
	Volume fraction (%)	9.2 ± 1.87	9.8 ± 1.84	7.3 ± 0.49
	Cell volume (μm ³)	1172 ± 64	1303 ± 274	1578 ± 95
	Elongation	2.73 ± 0.006	2.52 ± 0.066	2.51 ± 0.118
	Nuclei volume (μm ³)	325 ± 31	262 ± 45	193 ± 52
	Nuclei elongation	1.1 ± 0.03	1.18 ± 0.1	1.02 ± 0.12

KC	Age	P1	P16	Adult
	Total number/mm ³	107528 ± 17238	29785 ± 5343	18986 ± 3851
	Volume fraction (%)	8.8 ± 1.93	7 ± 1.15	3.6 ± 0.04
	Cell volume (μm ³)	2170 ± 386	1934 ± 246	1702 ± 86
	Elongation	1.7 ± 0.127	2.07 ± 0.120	2.34 ± 0.045
	Nuclei volume (μm ³)	352 ± 70	260 ± 42	151 ± 36
	Nuclei elongation	1.01 ± 0.09	1.36 ± 0.21	1.41 ± 0.3

Supp table 04: Morphometric analysis of BC and sinusoidal networks in absence of KCs

Bile canaliculi	Condition	PBS	Clodronate
	Total length (m/mm³)	4.94 ± 0.1	5.37 ± 0.2
	Volume fraction (%)	6.9 ± 0.72	5.66 ± 0.55
	Radius 2D (μm)	1.4 ± 0.11	1.2 ± 0.13
	Connectivity	0.88 ± 0.018	0.91 ± 0.012
	Branch length (μm)	8.3 ± 0.8	8.0 ± 0.3
	Junction density (number/μm)	0.06 ± 0.001	0.06 ± 0.002

Sinusoids	Condition	PBS	Clodronate
	Total length (m/mm³)	4.41 ± 0.3	3.87 ± 0.8
	Volume fraction (%)	22 ± 1.84	23.2 ± 4.76
	Radius 2D (μm)	3.3 ± 0.16	3.7 ± 0.69
	Connectivity	0.97 ± 0.012	0.96 ± 0.029
	Branch length (μm)	14.3 ± 0.1	13.6 ± 0.7
	Junction density (number/μm)	0.03 ± 0.001	0.03 ± 0.004

Supp table 05: Morphometric characterization of hepatocytes in absence of KCs

HEPATOCYTES (GENERAL)	Condition	PBS	Clodronate
	Total number/mm ³	302397 ± 26303	318449 ± 44948
	Volume fraction (%)	57.4 ± 1.86	57.7 ± 2.48
	Cell volume (µm ³)	3224 ± 260	3321 ± 461
	Mono-nuclear (%)	69.6 ± 0.8	51.4 ± 4.9
	Bi-nuclear (%)	24.3 ± 1.5	39.3 ± 4.9
	Nuclei volume (µm ³)	433 ± 58	436 ± 36
	Nuclei elongation	0.62 ± 0.068	0.49 ± 0.049

HEPATOCYTES (BY PLOIDY)	Ploidy	1 x 2n		1 x 4n		1 x 8n	
	Condition	PBS	Clodronate	PBS	Clodronate	PBS	Clodronate
	Percentage of total hepatocytes (%)	49.9 ± 3.8	31.9 ± 6.3	18.5 ± 2.8	17.1 ± 3.3	1.1 ± 0.2	2.1 ± 1.4
	Cell volume (µm ³)	1922 ± 86	1690 ± 172	3123 ± 143	2517 ± 161	3972 ± 329	4388 ± 530
	Elongation	0.73 ± 0.025	0.71 ± 0.035	0.68 ± 0.013	0.68 ± 0.066	0.57 ± 0.038	0.63 ± 0.047
	Apical surface (%)	21.3 ± 3.1	17.4 ± 1.7	24.5 ± 2.2	21.9 ± 1.8	28.4 ± 1.7	26.5 ± 1.3
	Basal surface (%)	35.1 ± 2.5	37.1 ± 4.1	38.7 ± 3.2	38.4 ± 6.1	35.6 ± 2.7	31.0 ± 8.5
	Lateral surface (%)	43.6 ± 5	45.5 ± 3.9	36.9 ± 4.6	39.7 ± 7.3	36.0 ± 2.0	42.5 ± 8.9
	Number of neighbours	8 ± 0.48	8 ± 0.78	9 ± 0.68	9 ± 0.93	11 ± 0.48	11 ± 1.04

Ploidy	2 x 2n		2 x 4n		2 x 8n	
Condition	PBS	Clodronate	PBS	Clodronate	PBS	Clodronate
Percentage of total hepatocytes (%)	15.3 ± 1.9	22.9 ± 3.6	5.4 ± 2.1	11.0 ± 3.1	0.4 ± 0.2	0.7 ± 0.2
Cell volume (µm ³)	4048 ± 126	3296 ± 354	6417 ± 504	5289 ± 817	7471 ± 1438	5175 ± 1918
Elongation	0.8 ± 0.003	0.82 ± 0.054	0.78 ± 0.056	0.79 ± 0.033	0.67 ± 0.082	0.88 ± 0.374
Apical surface (%)	25.9 ± 2.5	21.9 ± 0.9	30.5 ± 0.8	24.2 ± 1	26.4 ± 2.6	25.1 ± 4.2
Basal surface (%)	39.7 ± 2.8	42 ± 5.9	41.0 ± 5	40.9 ± 7.3	41.3 ± 1.5	39.9 ± 2.6
Lateral surface (%)	34.4 ± 4.8	36.2 ± 6.1	28.5 ± 4.2	34.9 ± 6.8	32.3 ± 3.6	35.1 ± 6.7
Number of neighbours	11 ± 0.84	10 ± 1.07	13 ± 1.07	12 ± 1.32	16 ± 1.11	12 ± 2.34

Supp table 06: Morphometric characterization of HSCs in absence of KCs

HSC	Condition	PBS	Clodronate
	Total number/mm³	40851 ± 11114	90995 ± 24403
	Volume fraction (%)	6.5 ± 1.29	9.4 ± 2.63
	Cell volume (μm³)	1334 ± 117	1263 ± 135
	Elongation	3.02 ± 0.191	2.49 ± 0.147
	Nuclei volume (μm³)	307 ± 35	308 ± 41
	Nuclei elongation	1.2 ± 0.097	0.95 ± 0.100

KC	Condition	PBS	Clodronate
	Total number/mm³	23657 ± 1854	-
	Volume fraction (%)	4.8 ± 0.37	-
	Cell volume (μm³)	1753 ± 290	-
	Elongation	2.44 ± 0.079	-
	Nuclei volume (μm³)	323 ± 35	-
	Nuclei elongation	1.2 ± 0.046	-

Supp table 07: Antibodies, dyes and reagents used for staining and experiments

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Dilution or concentration	Description
Primary antibodies	anti-Flk1 (goat polyclonal)	R&D System	AF644	(1:100)	Sinusoidal marker
	anti-CD13 (rat monoclonal)	Novus	NB100-64843	(1:500)	Canaliculi marker
	anti-F4/80 (rat monoclonal)	Abcam	ab6640	(1:400)	F4/80 + cells marker
	anti-Desmin (rabbit polyclonal)	Abcam	ab15200	(1:200)	Stellate cells marker
Secondary antibodies	Donkey anti-Goat Alexa Fluor 647	Invitrogen	A-21447	(1:1000)	
	Donkey anti-Rat CF 568	Biotium	20092	(1:1000)	
	Donkey anti-Rabbit Alexa Fluor Plus 647	Invitrogen	A32795	(1:1000)	
Small dyes	Phalloidin Alexa Fluor 647	Invitrogen	A22287	(1:1000)	Cell border
	Phalloidin Alexa Fluor 488	Invitrogen	A12379	(1:100)	Cell border
	DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen	D1306	(1:1000)	Nuclei
Reagent	Clodronate and control (PBS) liposome	LIPOSOMA	CP-010-010	(5mg/ml)	