

Temporal *in vivo* platelet labelling in mice reveals age-dependent receptor expression and conservation of specific mRNAs

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Short title: Temporal mouse platelet labelling

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Supplemental Methods

RNA isolation

RNA was extracted from fluorescent activated cell sorted (FACS; BD FACS Aria II, BD Bioscience; 70µm nozzle, 70 Ps) platelets, using the QIAGEN miRNAEasy mini kit (Cat. No. 217004), following manufacturer's instructions. Sample pellets were lysed in 500 µL of Qiazol at the time of harvest. A mixture of synthetic cel-miR-39-3p (Qiagen, 219600) and MS2 carrier RNA (Roche, 10165948001) was added in a volume of 200 µL QIAzol reagent. Following brief mixing and incubation at room temperature for 5 minutes, 140 µL of chloroform (Fisher Scientific, Cat. No. 12832224) was added and the solution was mixed vigorously for 20 seconds, and further incubated at room temperature for 5 minutes. Samples were then centrifuged at 13,500 x *g* for 15 min at 4°C. 280 µL of upper (aqueous) phase was carefully mixed with 420 µL of 100% ethanol and then applied to columns and washed three times according to the manufacturer's protocol. First, 700 µL of RWT buffer was added, the column centrifuged at 12,000 x *g* for 1 minute, and the flowthrough discarded. Then, 500 µL RPE buffer was added and the columns centrifuged at 13000 x *g* for 1 minute, and the flowthrough again discarded. 500 µL RPE buffer was again added, and the columns centrifuged at 13000 x *g* for 2 minutes. Flowthrough was again discarded. Columns were transferred to a dry collection tube and centrifuged at 15000 x *g* for 1 minute to dry. Finally, RNA was eluted by the addition of 35 µL of nuclease-free H₂O and centrifugation at 9000 x *g* for 1 min at room temperature into a fresh microcentrifuge tube. RNA samples were stored at -80°C prior to further analysis.

Reverse transcription

To generate cDNA for gene expression studies, 8 µL of RNA from each sample were combined with 2 µL of SuperScript VILO cDNA Synthesis Kit (Invitrogen, 11755-250) in 96-well plates, centrifuged and incubated on ice for 5 minutes before transferring to a thermocycler for the following protocol:

Stage	Temp (°C)	Time (min)
Mix	25	10
Hold	42	120
Melt	85	5
Hold	4	∞

RT-qPCR

For RT-qPCR measurements of mRNAs in platelets, the SYBR Select Master Mix (Applied Biosystems, 4472908) and custom-designed primers (Integrated DNA Technologies, see below) were used. Primers were designed to span exon-exon junctions if possible (not possible with *Itga2b*, *Gp9*, *Srgn*, *Gnas*, *Alox12*). Briefly, 2 μ L of 1:25 diluted cDNA was combined with 2.5 μ L SYBR Select Master Mix and 0.5 μ L of primer mix containing 2 μ M forward primer and 2 μ M reverse primer, resulting in a total volume of 5 μ L. A Bravo Automated Liquid Handling Platform (Agilent) was used to prepare the reactions in 384-well plates. All qPCR measurements were performed on a ViiA7 Real-Time PCR System (Applied Biosystems), using the following thermocycler conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Data was analysed using ViiA 7 Software (Applied Biosystems).

Data was analysed using ViiA 7 Software (Applied Biosystems) following a standard yield assumption of 100 \pm 5% achieved during the exponential phase. Interplate differences in RT and qPCR efficiencies were mitigated by placing all samples onto the same RT plate as well as the same qPCR plate, thus enabling identical reaction conditions for all samples. Finally old and new platelets were derived from the same 4 donor mice used in this experiment, enabling a paired comparison of old and young platelets in the same mice.

Primer sequences

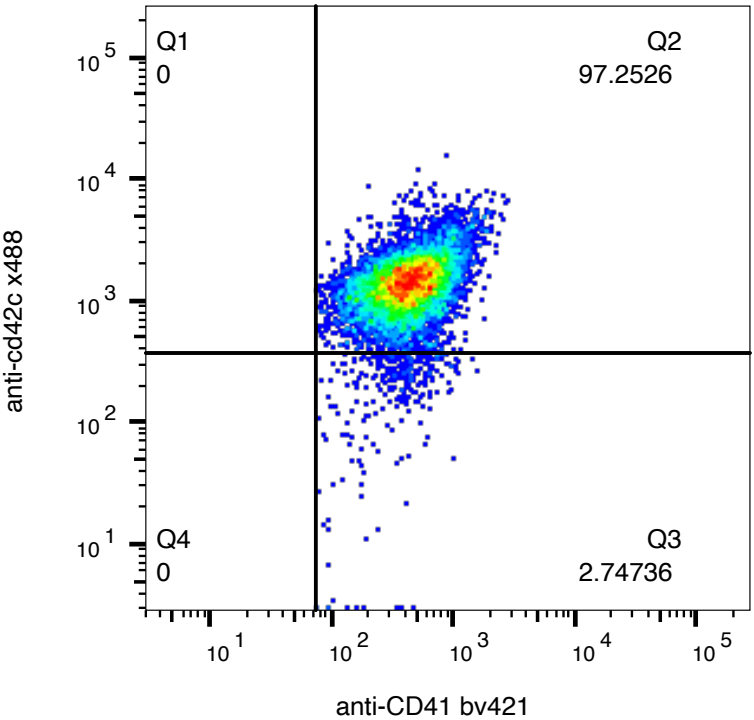
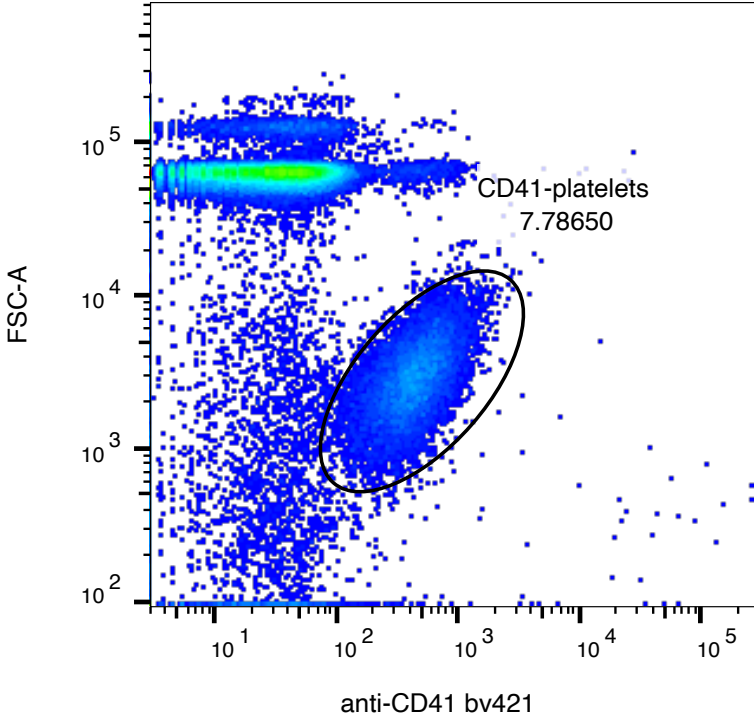
NCBI RefSeq	Target Gene	Description	Fwd Primer	Rev Primer
NM_007393	Actb	beta-actin	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG
NM_007440	Alox12	12-LOX	CAACCTAGTGCGTTTGTGGC	TCGGGAACGTGCGAAGTCAAA
NM_016745	Atp2a3	ATP 2a3 atpase	CCAGGAATCACATGGATGGTGT	TGGTATGGGGTCTGGAAGT
NM_009735	B2m	beta-2 microglobulin	ACCGTCTACTGGGATCGAGA	TGCTATTTCTTTCTGCGTGCAT
NM_007657	Cd9	CD9	CAGCGGAAACACTCAAAGC	TTCAGGGCAGGGCTTAACCT
NM_007687	Cfl1	Cofilin-1	TCCGGAAACATGGCCTCTG	AAAAGAGCACCGCCTTCTTG
NM_013885	Clic4	Chloride intracellular receptor 4	AGGCTAATGAAGCGCTGGAG	CATCTCATCGCCGTCCAGAA
NM_007976	F5	factor 5	CACGATCTAGCAACTCAGGACT	GGATGCTTAGTGGTTTGTCTGC
NR_003258	Gnas	G-protein alpha subunit	ACCTAAGGGTGCTGGAGAGT	TTCTCACCATCGCTGTTGCT
NM_018762	Gp9	glycoprotein 9	CTGTACCTGCCAGTCCTTGG	GCACTGAACGCAGGCTATTG
NM_010575	Itga2b	CD41/GPIIa	TCTTCAAGCGGAATCGACCG	ATGGAGTTCTGAGCAAAGGCA
NM_008410	Itm2b	integral membrane protein	CCTGGACAAGTGCTACGTGA	AGGTAGGTCCCAGCCTTAAT
NM_172118	Myl9	myosin light polypeptide 9	CTCTGCAGCAGGGAAACC	CATGGCGAAGACATTGGACG

NM_022029	Nrgn	neurogranin	ATGGA CTGCTGCACGGAGAG	CCGGAAACTCGCCTGGATT
NM_008771	P2rx1	p2x purinoreceptor	TACCATCGGCTCTGGGATTG	GTCACGTTACCCTCCCA
NM_001032378	Pecam1	CD31 PECAM	GAGCCTCACCAAGAGAACGG	CTCTTCTCGGGACATGGACG
NM_019932	Pf4	Platelet factor 4	TCCCGAAGAAAGCGATGGAG	CTTCAGGGTGGCTATGAGCTG
NM_023785	Ppbp	platelet basic protein	TGGGCTTCAGACTCAGACCT	ATGGGTCCATGCCATCAGATTT
NM_024457	Rap1b	GTP-binding protein	AAGGCCCGCAAAAAGTCATC	CAGTTCTTCAGACCTGGCTC
NM_022881	Rgs18	regulator of G- protein signaling 18	GAGGCCAAAATCAGAGCGAAA	AGCCCATTTCACTGCTTCTTCA
NM_080559	Sh3bgrl3	SH3 domain binding protein	CGGCTCCCGAGAAATCAAGT	CTCATCTCGAAGGGCGTTGT
NM_011157	Srgn	Serglycin	AGGAACCCAACTCGCATTGT	TCCAATGGCCACAGCTCAAT
NM_021278	Tmsb4x	thymosin, beta 4	CCTCTGCCTTCAAAAGAAACAAT	AAGAAGGCAATGCTCGTGGA
NM_001080971	Tubb1	tubulin 1	TGGGTTCAAGGCTAGGATGAG	TCACCTCCCAGAACTTGGCTC
NM_011738	Ywhah	14-3-3 epsilon	GCCATGAAGGCGGTGACAG	CCATCTGCCATGGTTTTCTGC

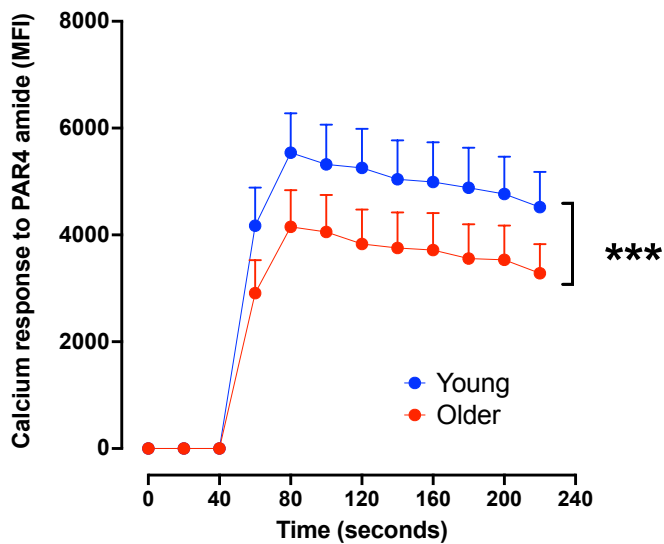
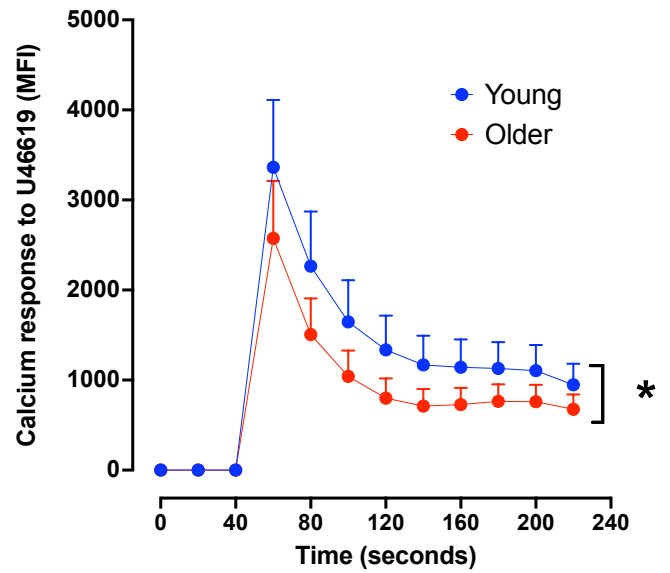
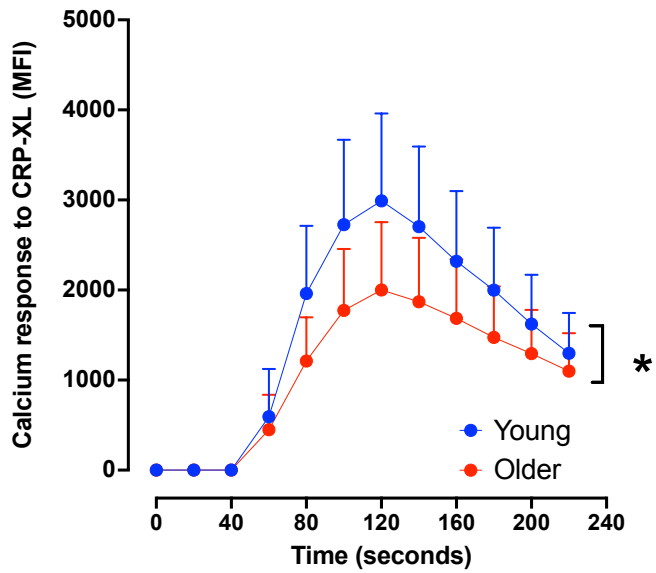
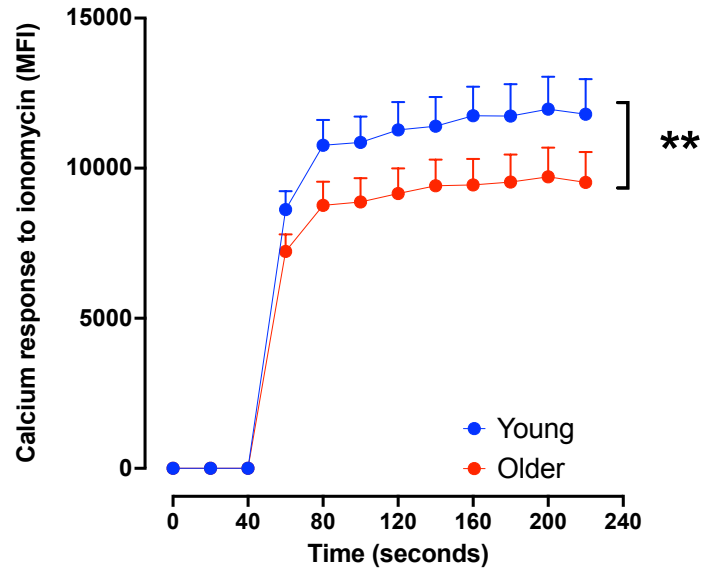
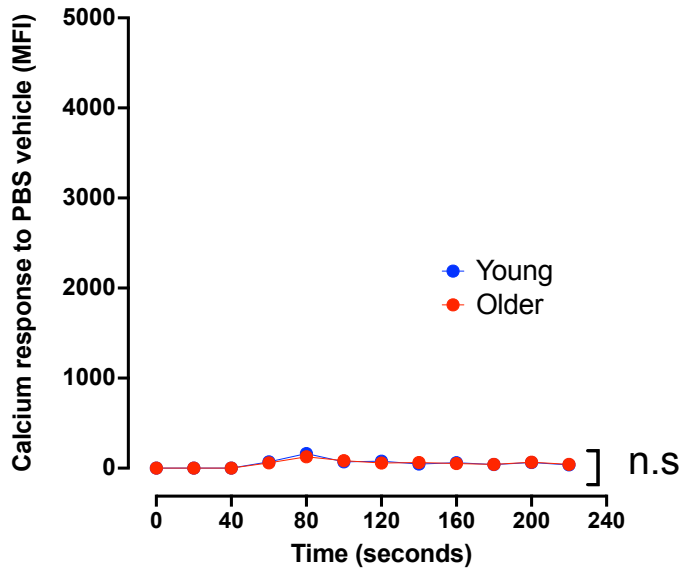
Supplemental Table 1

		ATP2A3	ALOX12	TUBB1	ITGA2B	GNAS	F5	PECAM1	YWHAH	CFL1	RGS18	P2RX1	ITM2B	CLIC4	ACTB	RAP1BP	MYL9	TMSB4X	NRGN	GP9	PPBP	SRGN	SH3BGRL3	CD9	PF4	B2M
Mouse 1	<24h	28.133	26.934	26.484	26.667	29.742	29.212	30.345	26.598	27.915	28.990	30.310	28.081	27.452	23.759	27.471	26.316	23.312	25.514	27.743	24.406	35.827	26.309	25.264	25.785	25.830
Mouse 2	<24h	26.938	25.626	25.238	26.123	28.689	27.426	29.463	24.907	25.865	27.633	28.629	26.823	26.160	22.620	26.161	24.954	21.799	23.858	26.297	22.018	32.475	25.319	24.013	24.033	24.689
Mouse 3	<24h	27.229	25.840	25.297	26.223	28.461	26.696	29.059	24.423	25.476	27.220	28.539	26.631	25.928	22.388	25.794	24.637	21.608	23.876	25.975	21.517	31.217	24.989	23.883	23.936	24.646
Mouse 4	<24h	26.801	25.439	25.249	26.021	28.992	26.691	29.079	24.375	25.288	27.010	27.816	26.281	25.574	22.224	25.775	24.481	21.340	23.720	25.698	21.188	32.243	24.991	23.540	23.457	24.206
Mouse 1	1-5days	34.166	32.395	32.331	32.309	33.602	35.299		31.984	31.559		34.624	31.603	30.728	27.470	32.693	29.548	27.624	28.975	30.977	28.651		29.178	27.656	28.523	28.943
Mouse 2	1-5days	32.033	31.299	29.778	31.022	35.016	31.287	34.027	29.583	30.425	31.811	33.624	31.766	30.440	26.894	29.885	29.176	25.965	28.113	30.170	25.453	34.772	28.168	26.906	26.455	26.922
Mouse 3	1-5days	34.213	32.029	31.691	31.855	33.897	32.466	35.136	29.041	31.109	33.000	33.205	31.124	30.492	26.865	29.553	28.558	25.088	27.621	29.296	24.177	34.911	27.981	26.265	25.665	26.451
Mouse 4	1-5days	32.096	30.356	30.229	31.587	34.478	31.952	33.716	29.156	30.435	31.144		31.073	30.368	26.421	29.467	28.980	25.047	27.875	29.384	24.212		27.812	26.421	25.993	26.272
Mouse 1	Global	30.693	29.263	28.927	29.204	32.095	30.179	33.171	27.946	29.026	29.973	31.643	29.125	29.820	25.683	28.593	28.131	24.284	27.220	29.316	25.381		27.836	26.233	26.256	26.534
Mouse 2	Global	28.863	28.171	27.760	28.071	30.804	29.998	31.697	26.923	27.895	30.208	31.046	28.983	28.029	24.603	28.053	26.886	23.619	25.940	28.128	23.564	34.994	26.947	25.498	25.161	25.654
Mouse 3	Global	29.634	28.375	28.230	28.981	31.491	29.299	31.423	26.902	27.875	29.966	30.644	29.135	28.590	24.725	28.074	26.990	23.712	26.094	28.392	23.248	32.798	26.931	25.755	25.386	25.836
Mouse 4	Global	29.150	28.556	28.064	29.121	31.713	29.409	31.843	27.537	28.648	30.386	31.753	29.504	28.221	25.077	28.534	27.271	24.026	26.638	28.171	23.769	33.589	27.415	25.996	25.577	26.268

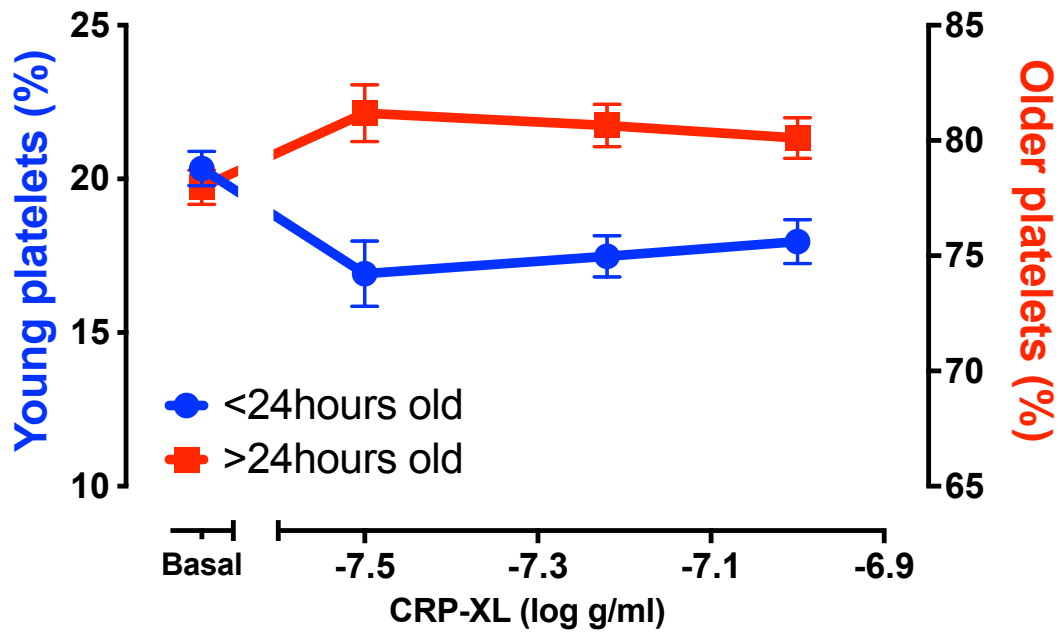
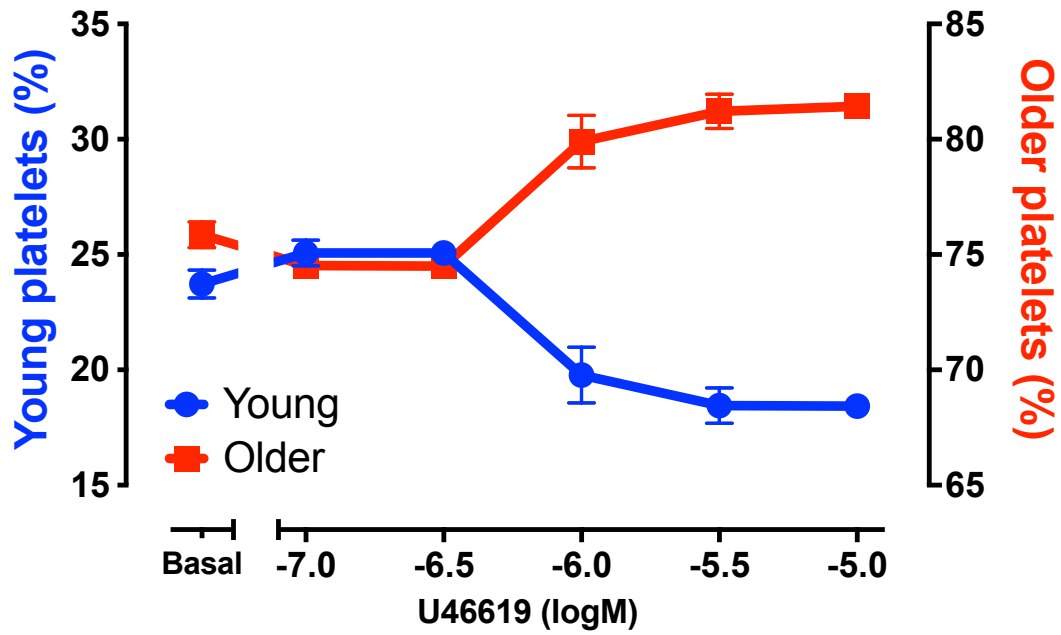
Supplemental Figure S1



Supplemental Figure S2



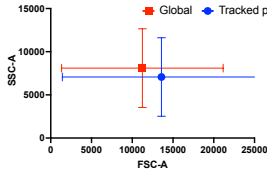
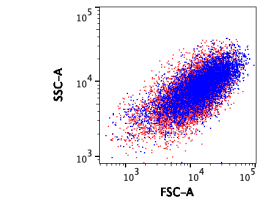
Supplemental Figure S3



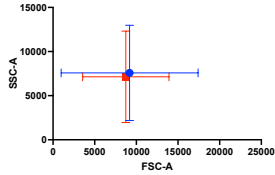
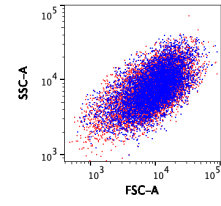
Supplemental Figure S4

Mouse 1

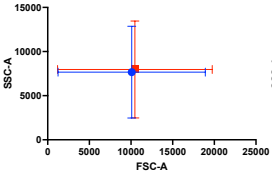
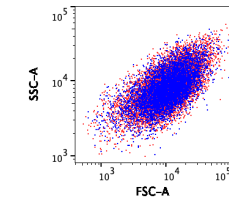
Day 1



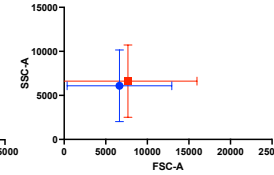
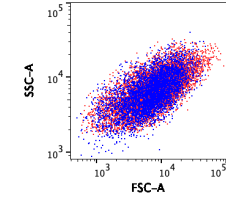
Day 2



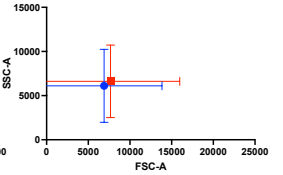
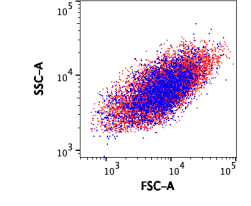
Day 3



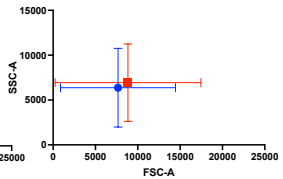
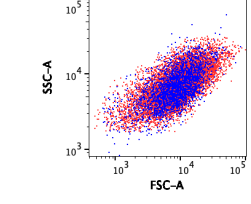
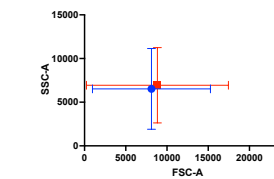
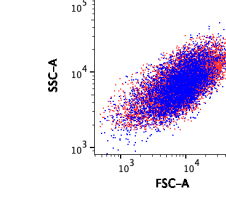
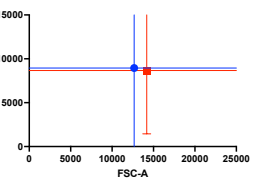
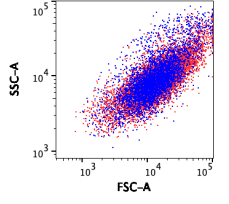
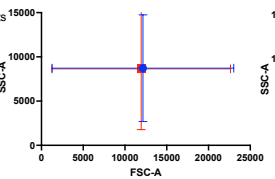
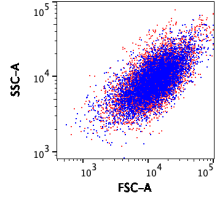
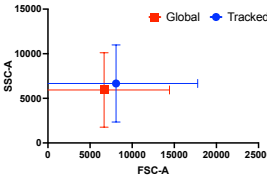
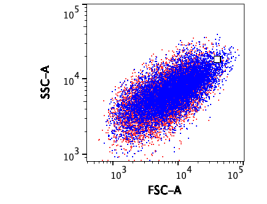
Day 4



Day 5



Mouse 2



Mouse 3

