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Belmonte, Miriam and Kent, David G orcid.org/0000-0001-7871-8811 (2021) Protocol to maintain single functional mouse hematopoietic stem cells in vitro without cell division. STAR Protocols. 100927. ISSN: 2666-1667

<https://doi.org/10.1016/j.xpro.2021.100927>

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Protocol to maintain single functional mouse hematopoietic stem cells *in vitro* without cell division

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Summary

This protocol details the isolation and *in vitro* maintenance of single hematopoietic stem cells (HSCs) in the absence of the bone marrow niche. The HSCs do not divide over 7 days and fully retain their long-term functional capacity in transplantation assays. Following hibernation culture, HSCs can be used to study quiescence exit and can be genetically manipulated as single cells prior to division.

For complete information on the generation and use of this protocol, including the single cell molecular profile of these cells, please refer to Oedekoven et al. (2021) (Oedekoven *et al.*, 2021).

Graphical abstract

Before you begin

Hematopoietic stem cells are thought to reside at the apex of the hematopoietic hierarchy and possess the unique capacity to both self-renew (perpetuating themselves as an undifferentiated population) and differentiate (generating the correct numbers and types of mature cell progeny to perform the necessary functions of the blood system). The development of clonal approaches to purify blood stem and progenitor cell populations has enabled significant breakthroughs in the field including the formal documentation of HSC functional heterogeneity and the development of a wide array of molecular tools to study HSCs at the single cell level. This has also led to a range of studies aimed at mimicking the HSC environment *in vitro* in order to develop protocols that would successfully expand hematopoietic populations in a dish.

In Oedekoven et al., we described a powerful culture system which maintains mouse and human HSCs as single cells, while preserving their functional and molecular properties, allowing a 7-day culture window to study HSCs in a distinct physiological context (Oedekoven *et al.*, 2021). These “hibernation cultures” keep HSCs from dividing by removal of key mitogens SCF and TPO from standard conditions. Optimization and utilization of such *in vitro* cultures would have a range of applications for manipulating HSCs at the single-cell level in both experimental and clinical research.

The protocol below describes the specific steps for using serum-supplemented medium. However, we have also validated the hibernation protocol in the serum-free culture media published in Wilkinson et al. (2019) (Wilkinson *et al.*, 2019) as reported in Oedekoven et al., 2021 (Oedekoven *et al.*, 2021).

Prepare washing buffer (PBS 2%FCS)

Timing: [10 minutes]

1. Mix all reagents listed below:

Reagent	Final concentration	Amount
PBS	-	490mL
Fetal Calf Serum	2%	10mL
Total	N/A	500mL

2. Filter using a 0.2µm filter and store at 4°C for up to 1 month

Prepare culture medium

Timing: [10 minutes]

3. To prepare 10 mL of medium (amount for loading 100µL/well of a 96-well plate), mix all the reagents listed in the table below except for IL-11 (which will be added to the plate after sorting the cells)

Reagent	Stock concentration	Final concentration	Amount (mL)
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StemSpan SFEM	-	-	8.78
Fetal Calf Serum	-	10%	1
Penicillin / Streptomycin	100 units/mL	1%	0.1
L-Glutamine	200mM	1%	0.1
10 ⁻⁴ M 2-Mercaptoethanol	-	0.2%	0.02
Interleukin 11 (IL-11)	10ng/mL	20 ng/mL	0.02

4. Filter the medium using a 0.2µm filter before use
 - i. Store the medium at 4°C for up to 1 week, if needed

Pre-load medium a U-bottom 96-well plate

5. Load 50µL of the culture media (described above) per well into U-bottom 96-well plate(s).

CRITICAL: Keep the rest of the medium at 4°C as this will be added after the cell sort using a 2X concentration of IL-11. This is to avoid the wastage of recombinant cytokines in the event that there is a technical failure at the cell sorter and to synchronize the stimulation time of the cytokine).

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45 FITC (dilution 1:100)	BD Biosciences	clone 30-F11
Anti-mouse EPCR PE (dilution 1:100)	STEMCELL	clone RMEPCR1560
Anti-mouse CD150 PE-Cy7 (dilution 1:100)	Biolegend	clone TC15-12F12.2
Anti-mouse CD48 APC (dilution 1:100)	Biolegend	clone HM48-1
Anti-mouse Sca-1 BV421 (dilution 1:200)	Biolegend	clone D7
Chemicals, peptides, and recombinant proteins		
PBS	Sigma	Catalog #D8537
Fetal Calf Serum (FCS) <i>FCS should be screened for high efficiency haematopoietic colony growth</i>	Sigma	Catalog #F7524
StemSpan	STEMCELL Technologies	Catalog #09650
Ammonium Chloride (NH ₄ Cl)	STEMCELL Technologies	Catalog #07850
2mM L-Glutamine	Life Technologies	Catalog #25030081
2-Mercaptoethanol	Fisher Scientific	Catalog #11528926
Penicillin and Streptomycin (Pen/Strep)	Sigma	Catalog #P4333
7-Aminoactinomycin D (1mg/mL solution in DMSO)	Life Technologies	Catalog #A1310
Recombinant mouse IL-11	Bio-technique	Catalog #418-ML
Critical commercial assays		
EasySep Mouse Hematopoietic Progenitor Enrichment kit	STEMCELL Technologies	Catalog # 19856
EasySep™ Magnet	STEMCELL Technologies	Catalog # 18000
Experimental models: Organisms/strains		

C57BL/6 mice, 8-12 weeks old	Charles River Laboratories	N/A
Other		
Dissecting instruments (scissors and forceps)	N/A	N/A
Mortar and pestle	N/A	N/A
50µm filters	Wolf Laboratories	Catalog #04-0042-2317
5 mL polypropylene tubes	VWR International	Catalog #352063
5 mL polystyrene tubes	Scientific Laboratory Supplies	Catalog #352054
50 mL conical tubes	VWR International	Catalog #525-0402
U-bottom 96-well plates	Scientific Laboratory Supplies	Catalog #353077

Step-by-step method details

Mouse bone marrow cell preparation

Timing: [approximately 2-3 hours, depending on the number of animals/samples]

This section describes the preparation of murine bone marrow cell suspension and magnetic bead depletion of progenitor cells (optional) by negative selection using the EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit (STEMCELL).

1. Harvest the bone marrow cells from femora, tibiae and pelvic bones removed from the mice by crushing the bones in 10mL PBS 2%FCS using a mortar and pestle. For specific animals where maximal cells are required, BM can also be obtained from the spine and forearms
2. Transfer the cell suspension into a 50 mL conical polypropylene centrifuge tube
3. Centrifuge the cell suspension for 5 minutes at 300 x g at room temperature
4. Remove the supernatant and resuspend the cells in 3mL PBS/FCS
5. Lyse the red blood cells by adding 5 mL Ammonium Chloride (NH₄Cl, STEMCELL) incubating the cells on ice for 10 minutes, vortexing after the first 5 minutes
6. Fill the tube up to 50mL with PBS/FCS and centrifuge for 5 minutes at 300 x g at room temperature
7. Resuspend the cells in 500µL/mouse PBS/FCS and transfer into a polystyrene round-bottom tube
 - i. Add 1mL of PBS/FCS to the empty 50mL conical polypropylene centrifuge tube from step 6 and use these leftover cells for preparing control samples of non-depleted cells. This is important to assist the user in setting gates for sorting as well as for determining the absolute frequencies of subpopulations of interest, since these cannot be measured from the enriched sample. Filter the suspension using a 50-µm filter. Use 100µL of this suspension into each of the FACS control tubes (unstained, 7AAD only, FITC only, PE only, APC only, PE-Cy7 only, BV421 only)

Optional: Perform lineage depletion by using EasySep mouse hematopoietic progenitor cell enrichment kit (STEMCELL). The lineage depletion step will reduce sorting time and reagent costs, especially when isolating HSCs from multiple mice. We typically use the Hematopoietic Enrichment Cocktail and EasySep technology from STEMCELL Technologies, however, other commercial suppliers of lineage depletion kits exist. Alternatively, the lineage depletion step could be replaced using directly conjugated antibodies which allow the exclusion of the differentiated population during the cell sorting (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119). This, however, will increase sorting time and reagent costs, compared to performing the lineage-depletion step prior to cell sorting.

Manufacturer protocol was followed with minor modifications to prioritize yield over purity in this pre-sort “debulking” step:

- Add 10 μ L/mouse EasySep Mouse Hematopoietic Progenitor Cell (HSPC) Isolation cocktail and incubate for 15 minutes, on ice.
- Add 15 μ L/mouse EasySep Streptavidin RapidSpheres and incubate for 15 minutes on ice
- Add PBS/FCS to a total of 2.5mL
- Insert the tube into the EasySep Magnet and incubate for 3 minutes at room temperature
- Transfer the supernatant into a new tube and repeat the magnet incubation step one additional time
- Centrifuge the cell suspension for 5 minutes at 300 x g at room temperature
- Resuspend the cells in 100 μ L/mouse PBS/FCS and transfer into a polypropylene tube to perform antibody staining

Antibody staining

Timing: [40-50 minutes]

This section describes the incubation of bone marrow samples with fluorescence-tagged antibodies and the preparation of compensation controls.

8. Add the following antibodies to the FACS control tubes:

Reagent	Label	Dilution
CD45	FITC	1:100
CD48	APC	1:100
CD150	PE-Cy7	1:100
EPCR	PE	1:100
Sca-1	BV421	1:200

9. Add all antibodies at the concentration specified above to the sample tubes (making an antibody master mix is recommended if more than one sample)
10. Vortex the cells in each tube and incubate on ice in the dark for 30 minutes
11. Wash the cells by adding 1mL of PBS/FCS to each tube and centrifuging for 5 minutes at 300 x g at room temperature
12. Resuspend the control tubes in 300 μ L PBS/FCS and add 1:1000 7AAD to the 7AAD-only control tube
13. Resuspend the sample tube in 500 μ L/mouse 1:1000 7AAD in PBS/FCS and filter the suspension using a 50- μ m filter into a new polypropylene tube. Add 500 μ L 1:1000 7AAD in

PBS/FCS to the original tube to make sure to rinse out the remaining cells and transfer them to the new tube through the 50- μ m filter

Sorting of ESLAM Sca-1^{high} LT-HSCs

This section describes the workflow to isolate single HSCs using fluorescent-activated cell sorting (FACS).

14. Use the unstained control to set voltages of each channel on the flow sorter and use 7AAD-only control to set the viability gates. Use the single stain controls to set up the compensation for each detection channel

Optional: Compensation beads can substitute for single stain controls, but it is important to remember that they will not account for background staining or autofluorescence and it is advisable to check the full panel in an initial setup phase.

15. Apply the compensation settings to the sample tubes
16. Load the sample tube on the flow sorter and set gates as shown in Figure 1
20. Use the single-cell deposition unit of the sorter to place 1 cell into each of the wells of U-bottom 96-well plates, each well having been preloaded with 50 μ L media (described above in “Before you begin” section)
21. Load 50 μ L media (described above) supplemented with a 2X cytokine concentration to bring the final medium to 1X cytokines

Pause point: The cell suspension can be stained for longer than 30 minutes on ice, but it is recommended not to exceed ~1hour in order to avoid overstaining the samples and increasing background signal. For a quicker preparation of single stain controls, tubes can be incubated for a shorter time (~15 minutes) at room temperature, but this is not advisable for primary samples where room temperature incubation may alter the function of cells.

Optional: Given the long preparation time required to obtain cell suspension pre-sorting, bone marrow harvest can be performed the day before the sort and the cell suspension can be stored in 10%FCS/PBS at 4°C overnight. If considering this option, consider storing the sample in a larger liquid volume (~5mL/mouse) and then spinning at 300 x g for 7 minutes followed by addition of crude DNase I to the pellet in the morning before undertaking lineage depletion and/or antibody staining described above.

Expected outcomes

Mouse bone marrow cell preparation

Cell suspension obtained from normal mice should look pinkish and be a bit cloudy. After the first centrifugation step the cell pellet will look reddish, but following the RBC lysis step and centrifugation, the supernatant should look clear and the pellet will be whitish, with red edges.

Preparation of cells for cell sorting

The final cell suspension should be contained in a volume of ~1mL/mouse processed and filtered before sorting, as described above, to remove any clumps of cells which might clog the flow instrument. When resuspended in 2%FCS/PBS, the cell suspension should look translucent.

Sorting of ESLAM Sca-1^{high} LT-HSCs

Representative flow cytometric plots for a typical ESLAM Sca-1^{high} cell sorting procedure are shown in Figure 1. Viable cells are gated based on their FSC and SSC properties and their exclusion of 7AAD. CD48⁺ cells are excluded by gating the bottom of the three major populations. These cells are then visualized in a CD45/EPCR plot and the double positive cells are selected. CD150⁺CD48⁻EPCR⁺Sca-1^{high} cells are then selected, which represents ~0.003% of the whole bone marrow cells in an adult C57BL/6 mouse.

Single-cell culture of HSC

In order to visualize the cells under the microscope, adjust the field of view to look at the entire well, then focus on the center of the well by using the coarse adjustment. In 96 well U-bottom plates, this is where the cell typically settles a few hours after sort. To facilitate the visualization of the single cells under the microscope, the plate can be spun in a centrifuge for 5 minutes at 240 x g to allow the cells to be collected at the center of the well (cells may not appear as spherical immediately after spinning). Alternatively, to facilitate visual identification, the first well can be used to sort 100 viable cells to allow establishment of the field of view for subsequent wells. Between 20 and 40% of single cells will survive 7 days of culture in the hibernation condition, compared to ~98% survival in 300ng/mL SCF-supplemented media. ~99% of the input HSCs cultured in the hibernation condition will not divide during the 7-day culture period and will be maintained as single cells (Figure 2-3).

Single cells for future manipulation

In Oedekoven *et al.*, 2021, we showed that single HSCs could have virus delivered to them without undergoing division (Oedekoven *et al.*, 2021). However, there are a wide range of other studies that could be imagined, including monitoring symmetric versus asymmetric partitioning of cellular components using high resolution imaging (Filippi, 2021; Wang *et al.*, 2021), single cell immunostaining (Ema *et al.*, 2007), or screening of compounds for improved survival or induction of cell division.

60-70% of single HSCs cultured in the hibernation condition for 7 days are able to generate clones in colony forming cell assays at day 14, with the majority generating at least three different mature cell types (as determined by flow cytometry for cell surface markers Ly6g (Gr-1), CD11b (Mac-1), Ter-119 and CD41) (Figure 4).

Limitations

Low survival in hibernation culture

HSC survival in hibernation culture is decreased in the absence of SCF compared to SCF-supplemented medium, as shown in Figure 2. Further optimization of the hibernation culture and/or the addition of additional supplement (e.g., Rho kinase inhibitors (Claassen, Desler and Rizzino, 2009), fibronectin, PVA (Wilkinson *et al.*, 2019)) could improve the survival *in vitro* and allow larger-scale studies, without requiring the use of larger number of animals to obtain higher cell output.

Potential selection of a specific HSC phenotype

In Oedekoven et al. (Oedekoven *et al.*, 2021), we investigated whether differences in HSC subtypes (in accordance with Dykstra et al., 2007 (Dykstra *et al.*, 2007)) were observed between hibernating and freshly isolated HSCs in single cell transplantation experiments. Although it would need to be confirmed in a larger number of recipients, the current data suggest that hibernation conditions might preferentially retain α -HSC (Oedekoven *et al.*, 2021) which is also consistent with the higher levels of CD150 expression previously reported (Beerman *et al.*, 2010; Morita, Ema and Nakauchi, 2010). This could indicate that the α -HSC subtype is more resilient in the hibernation culture and that the cytokine deprivation might be selecting for the more myeloid-biased HSCs and this property may also be related to the delayed engraftment that is observed in α -HSCs.

Long-term survival of HSCs in hibernation culture

Cell survival decreases overtime, and reduces after 7 days of culture, making longer term cultures in this current condition challenging (Figure 5). We have tested up to 10 days in some experiments without observing an additional decrease in cell survival, however, we are unable to say whether extended culture beyond 10 days would also permit the same subset of HSCs to survive.

Proliferation of HSCs in hibernation culture

Our data show that over 99% of HSCs cultured in hibernation condition remain as single cells over the 7-day hibernation culture. However, a small fraction of single cells (~0.8%) do undergo division. In order to confirm that none of the cells which survived on day 7 have undergone division followed by death of one daughter cells, we recommend performing daily counts or time-lapse imaging to monitor potential cell division/cell death.

Gene expression changes

Gene expression profiling of single LT-HSCs in 7-day hibernation conditions revealed a high retention of G0/G1 cell cycle genes, as well as self-renewal regulators of the HSC-related MoIO signature (Wilson *et al.*, 2015), suggesting that such genes are indeed likely to be essential for HSC function and confirming the functional HSC identity of hibernating HSCs at the transcriptional level. However, some molecular differences between hibernating and freshly isolated HSCs were observed, and a number of regulators were not expressed in hibernating HSCs (e.g., members of the AP1 complex). These observations suggest that the hibernation cultures might drive the reduced expression of these factors. That said, it is important to note that SCF-stimulation of the hibernating HSCs did not re-initiate their expression, potentially disfavoring this hypothesis (Oedekoven *et al.*, 2021).

Troubleshooting

Problem 1:

Insufficient time on the sorter to obtain the required cell number and/or insufficient number of cells obtained after sorting.

Potential solution:

Insufficient time/cells might be caused by insufficient number of input cells. Consider harvesting more bone marrow (more mice) and performing the optional pre/sort lineage depletion (e.g., using EasySep Lineage Depletion cocktail from STEMCELL Technologies). This step will help decreasing the number of contaminating cells and will increase the absolute yields. If performing single cell sorting, it is advisable not to increase the speed of cell sort beyond ~2000 cells/s in order to avoid too many aborted decisions in the single cell/droplet setting on the flow sorter.

Problem 2:

High number of 7AAD-positive cells at the sorter.

Potential solution:

Depending on the final panel of antibodies/fluorochromes used, there may be some compensation issue causing cells to appear 7AAD positive. This is most likely to be the case if these cells are directly above the viable cell fraction in FSC (as events lower in FSC are likely to be dead). Check with a flow operator and adjust the flow cytometer's settings and compensation where necessary. If the cells were indeed dead, some possible approaches can be taken including keeping the cells and plates on ice and not leaving cells overnight. It would also be worth checking reagents to ensure that they are at the correct concentrations and not inadvertently driving cell lysis (e.g., 10X PBS versus 1X PBS).

Problem 3:

Difficulty finding the single cells in individual wells under the microscope.

Potential solution:

There are two possible issues: 1) the cells were not sorted properly or 2) the cells are present, but you cannot find them. If the cells were not sorted correctly in the wells, check with the flow operator the flow cytometer's settings and calibration used (including sort reports) and consider performing a set of optimization experiments with cells (not beads) to ensure that the single cell deposition unit is functioning properly. If the cells are there, but you cannot find them, another possible explanation is that the medium is full of debris which make hard to distinguish the cells. We recommend always filtering the media before sorting into it, by using a 0.22µm filter and to sort ~100 viable cells into well A1 to allow efficient finding of the focal plane for visualizing cells. We also suggest to always prepare a plate loaded with media (as above) with 300ng/mL Stem Cell Factor (SCF), as good control for the sorter/sorting. In this latter scenario, we would expect to obtain over 90% survival and cell division to occur following 24-48 hours (Kent *et al.*, 2013).

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David G. Kent david.kent@york.ac.uk.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data sets supporting this protocol, and used in Figures, have not been deposited in a public repository but are available from the corresponding author upon request.

Acknowledgments

We thank all of the authors of the original study (Oedekoven *et al.*, 2021) from which this protocol was generated. We thank Reiner Schulte, Chiara Cossetti, and Gabriela Grondys-Kotarba of the CIMR Flow Cytometry core for technical assistance and suggestions, Sally Thomas and the Central Biomedical Services unit staff, and Mairi Shepherd for providing some of the illustrations contained in the graphical abstract. Work in the D.G.K. laboratory is supported by an ERC Starting Grant (ERC-2016-STG-715371), an MRC-AMED joint award (MR/V005502/1), a CR-UK Programme Foundation Award (DCRPGF\100008), and a core support grant to the Wellcome MRC Cambridge Stem Cell Institute.

Author contributions

M.B. analyzed the data reported in this study and generated the graphs and figures. M.B., and D.G.K. wrote the manuscript.

Declaration of interests

The authors declare no competing interests.

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Figure legends

Figure 1: Representative gating strategy to purify ESLAM Sca-1^{high} LT-HSCs. On a SSC-A vs FSC-A plot, gate a region which excludes most of the dead cells and debris and very large cells. Gate on single cells using pulse width and FSC-A as shown. Exclude dead cells by gating 7AAD-negative cells. To set the SLAM population, gate CD48⁻ CD150⁺ cells, being careful not to go too high into the CD48^{dim} population. From the SLAM population, gate CD45⁺EPCR⁺ cells with a relatively strict EPCR⁺ gate (initially described as EPCR⁺⁺)(Kent *et al.*, 2009). To set the ESLAM Sca-1^{high} population which has an increased proportion of LT-HSCs but reduced overall yield, gate Sca-1^{high} cells (described in Wilson *et al.* 2015 (Wilson *et al.*, 2015)).

Figure 2: Adapted from Oedekoven *et al.* (Oedekoven *et al.*, 2021). Left panel: HSC survival in hibernation conditions is decreased compared to SCF-supplemented medium (+SCF n=355, 5 biological replicates; -SCF n=1722, 7 biological replicates). Right panel: number of clones that had divided (numbers of wells with >2 cells were scored to determine) at day 7 post-isolation. Only culture conditions without SCF retained HSCs as single cells. Bars show mean with SEM. Unpaired t-test: *p<0.05, **p<0.01, ***p<0.001.

Figure 3: Time-lapse image of single HSCs in hibernation cultures. The three pictures show an individual well imaged on day 1, 3 and 7 of culture in the hibernation condition. Single cells do not divide over the course of the 7-day hibernation culture. Scale bar represents 50um. See also the supplemental video in Oedekoven *et al.* (Oedekoven *et al.*, 2021)

Figure 4: Single HSCs in hibernation culture retain multi-potency. Adapted from Oedekoven et al. (Oedekoven *et al.*, 2021). Left panel: Colony forming cell assay efficiency for single HSCs in hibernating culture (n=230, 6 biological replicates). Right panel: ~80% of single cells generate colonies of at least three lineages in colony forming cell assays (n=166, 3 biological replicates). Colonies are defined as MK (containing cells positive for CD41, a megakaryocyte marker), GM (containing cells positive for granulocyte/monocyte markers Gr-1 and CD11b), GEM (containing cells positive for granulocyte/monocyte markers Gr-1 and CD11b), GMM (positive for GM and MK markers), and GEMM (positive for GM, MK and erythrocyte markers). Bars show mean with SEM.

Figure 5: SCF-deprivation decreased HSCs survival in culture compared to SCF-supplemented conditions, and only ~25-35% remain viable single cells up to 7 days in culture (+SCF, 5 biological replicates; -SCF, 5 biological replicates). Bars show mean with SEM.

Figure 1

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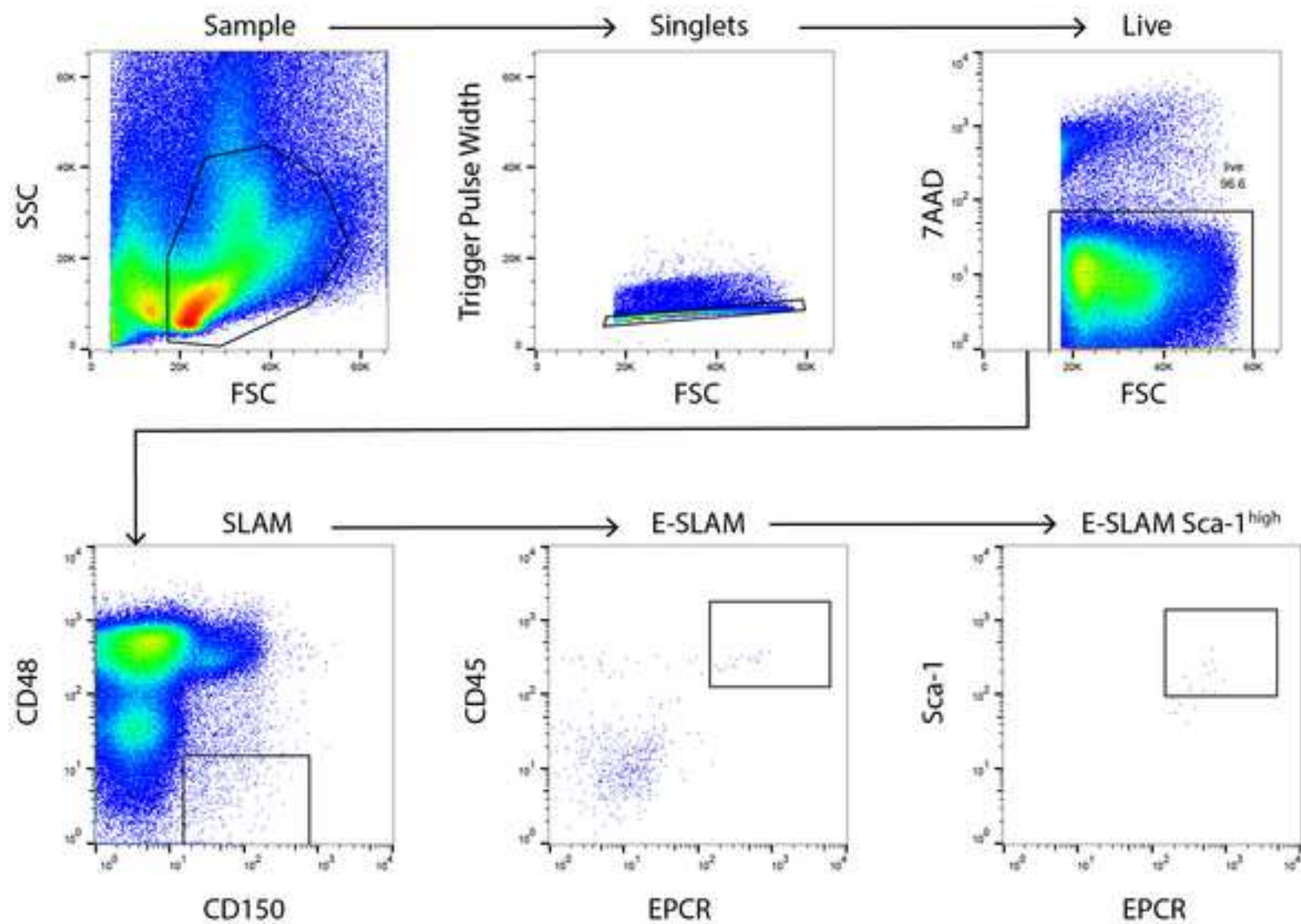
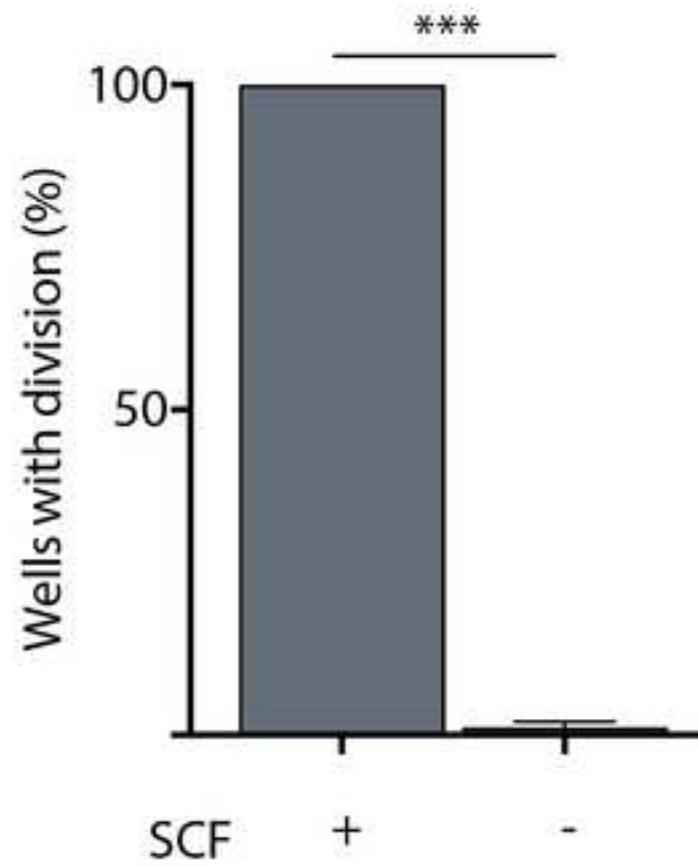
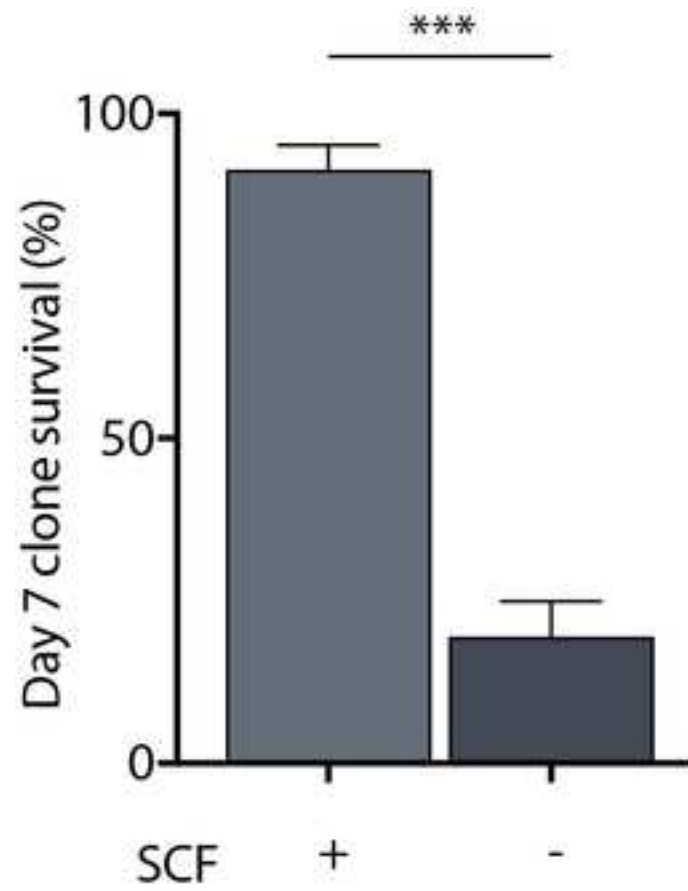


Figure 2



Day 1



Day 3



Day 7



Figure 4

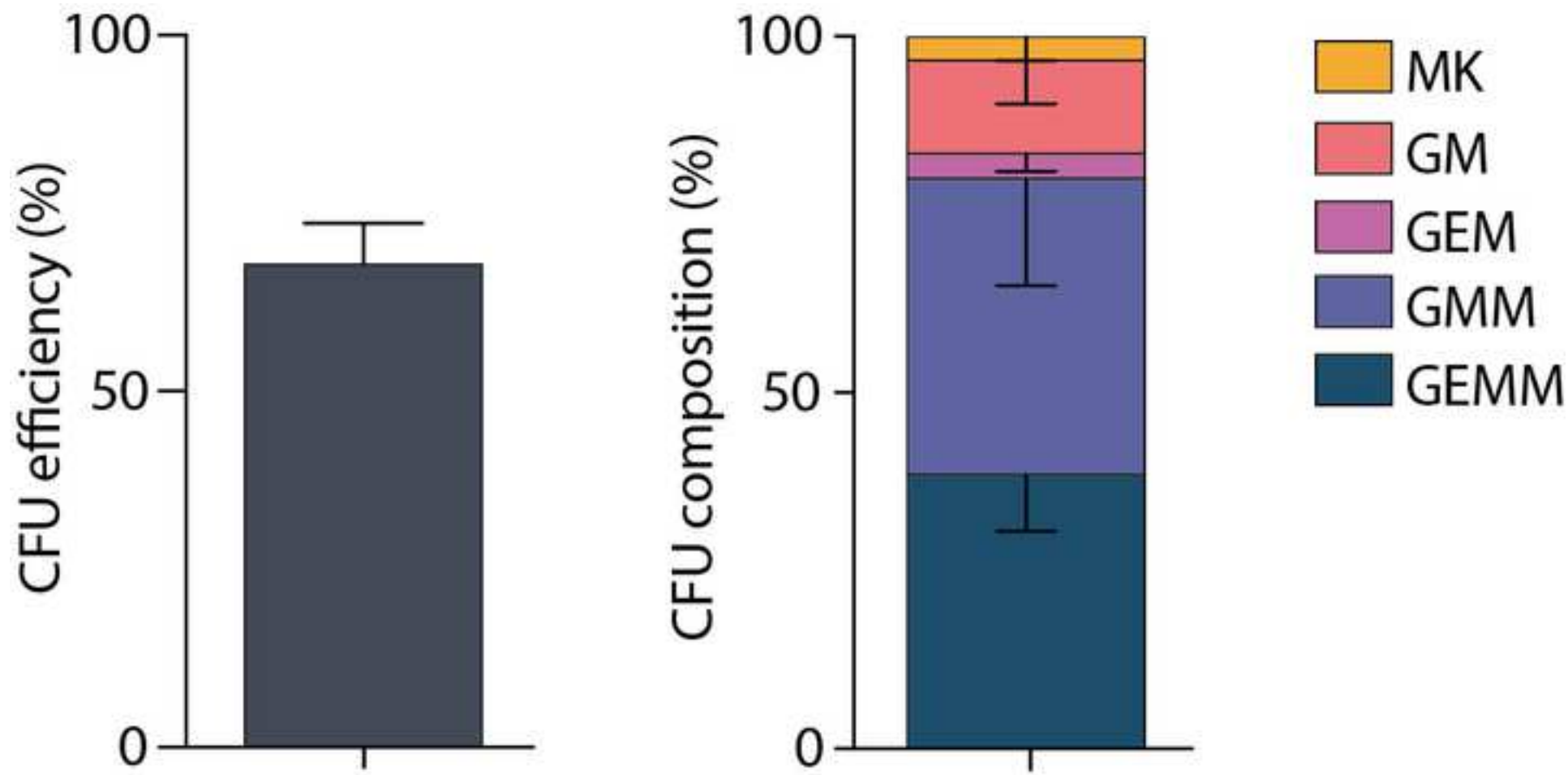


Figure 5

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