

Protocol for preparation of mouse liver Kupffer cells and liver sinusoidal endothelial cells

Developed and used in the Vascular Biology Research Group at the University of Tromsø, Norway. Please direct questions and comments to:

Bård Smedsrød

Professor and Head of Vascular Biology Research Group (www.uit.no/research/vbrg)

Scientific head of Laboratory for Electron Microscopy (www.uit.no/forskning/em)

Department of Medical Biology

University of Tromsø

NO-9037 Tromsø

NORWAY

tel.: +47-77644687 / +47-99599463

e-mail: bard.smedsrod@uit.no

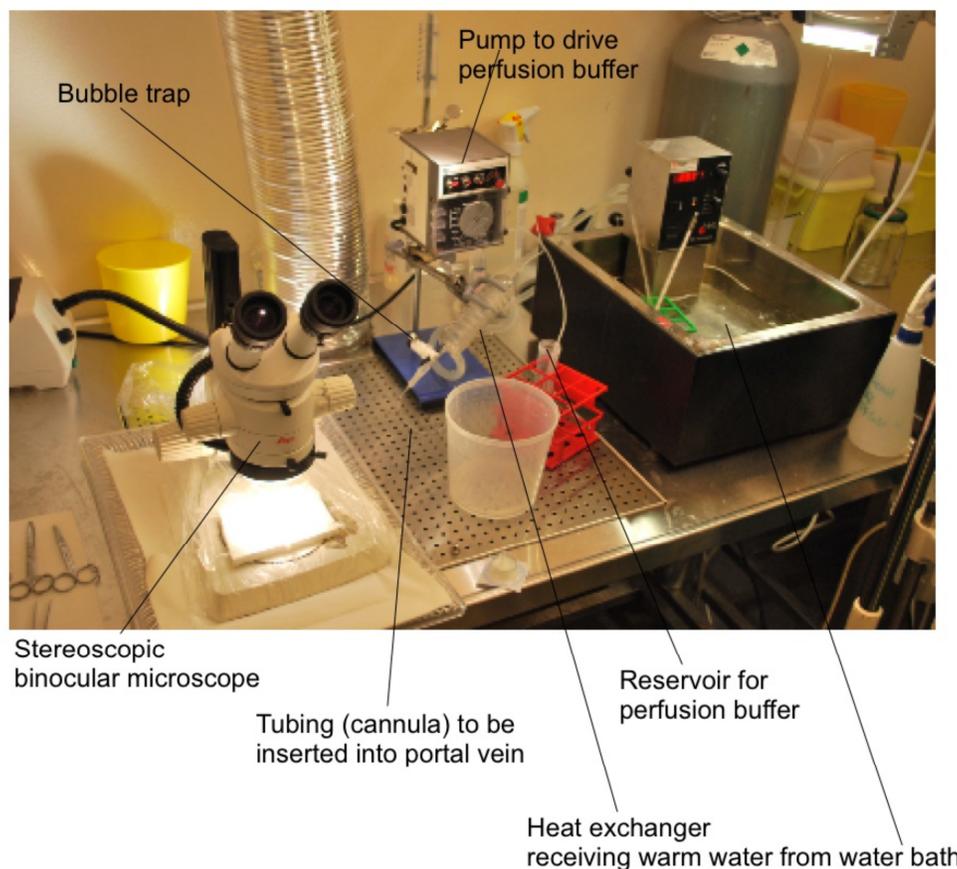
The following protocol is an outline of the method we use to prepare Kupffer cells (KC) and liver sinusoidal endothelial cells (LSEC) from mouse liver. The method is based on the one originally published to prepare rat liver KC and LSEC (1), and is slightly modified from the one outlined in (2). We also strongly recommend reading our 2012 review paper (3) to obtain a solid background understanding of the physiological functions of the LSEC.

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BASIC HARDWARE

- Water bath
- Condenser/heat exchanger
- Pump
- Silicone tubing (diameter varies in different part of the circuit, we use adaptors to prevent leakage)
- Bubble trap
- Surflo-W catheter (0.64 x 19 mm) (Terumo)
- Magnifier/stereoscopic binocular microscope (not compulsory, but makes it easy to see the vein and cannulate properly. It also allows observing the canula all the time which is very important to monitor if bubbles enter the system)



TIP:

- The temperature of the water bath needs to be adjusted to give the buffer 37°C at the point where it leaves the canula. (Tubing diameter, distance from heat exchanger, and flow rate affect the temperature loss along the tubing.)

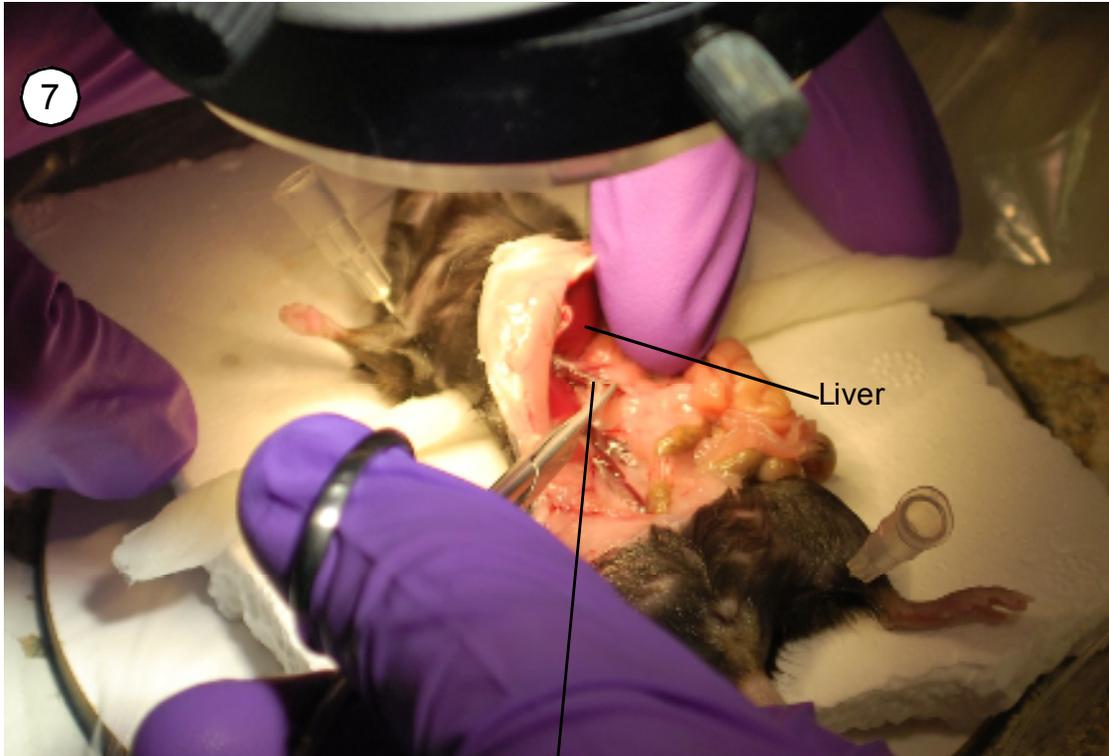
MATERIALS FOR LIVER PERFUSION

- Small styrofoam “bed” for the mouse + “pillow” (paper rolled to form a pillow)
- Good scissors, forceps, surgical tweezers (preferably with blunt edge)

- Perfusion buffer
- Dissociation buffer (enzyme buffer)
- Preservation buffer
- Falcon 35-3004 petri dish
- Fridge/ ice box for buffers

PROCEDURE FOR LIVER PERFUSION

1. Preparing the system:
 - Wash the system with at least 50 ml perfusion buffer.
 - Allow the water bath to attain the right temperature.
 - Empty bubble trap, make sure there are no bubbles in the system.
 - Flow rate before starting: 7 ml/min.
 - Make sure there is 50 ml perfusion buffer in the tube before starting. (NB: more than one tube may be needed for one perfusion.)
2. Anesthetize or kill the animal.
3. Place the animal on the bed and tape/pin the limbs so the mouse stays as straight as possible. Place the paper pillow under the abdomen, so the liver will be higher than the rest of the organs. (The pillow should neither be too thick nor too thin; it has to be thick enough for the liver and the portal vein to come up, but not too much as this will affect the perfusion).
4. Open the animal to expose the peritoneal cavity carefully so as not to break the liver. The abdomen cavity has to be as exposed as possible for better access and visualization.
5. Place the digestive system on the side to expose the portal vein.
6. Reduce the flow to 3 ml/min (make sure that you have enough buffer in case more time is needed for the operation). NB: it is important to maintain this low flow rate during the insertion of the cannula in order to avoid rupture of the portal vein. Never turn off the flow rate completely during the operation.
7. (*See next page for illustration of step 7*) Make sure you have the microscope focused on the portal vein and make an incision (about 1/3 of the vein diameter). NB: do not cut completely the portal vein, as this will make it very difficult to insert the cannula). The incision should be made close to the where the splenic vein is entering the portal vein. In this way one ensures that the distance from the incision to the point where the portal vein enters the liver is sufficient to give the cannula a stable position, and at the same time the tip of the canula will rest in a position to allow all the lobes to get perfused. Carefully lifting up the stomach helps to monitor the portal vein as the cannula is inserted.
8. Insert the cannula, parallel to the level of the vein. In this way the cannula will slide smoothly in, and the risk of rupturing the vessel wall is minimized. (A certain sign that the canula is inside is when the color of the liver changes from dark red to yellow.)
9. Cut the inferior vena cava to allow the perfusion buffer to flow freely through the liver.
10. Increase the flow up to 7-10 ml/min depending on the animal weight.
11. Keep on perfusing the liver with perfusion buffer until it is clean from blood, (normally takes 10-30 ml).
12. Change to the dissociation buffer (50 ml) (The liberase is kept frozen until needed. Keep pipettes and tips available.)

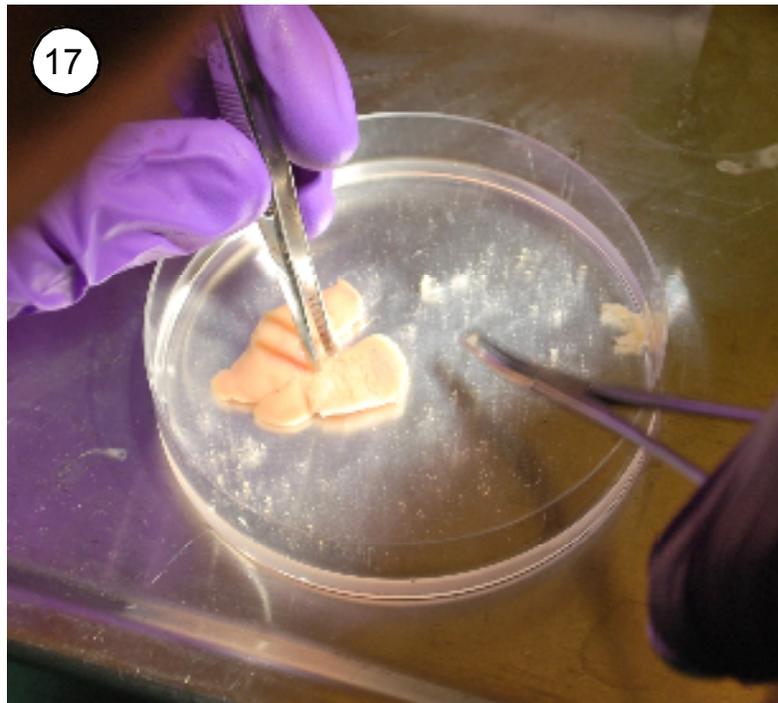


Incision is cut 1/3 through the portal vein to allow insertion of tubing/cannula



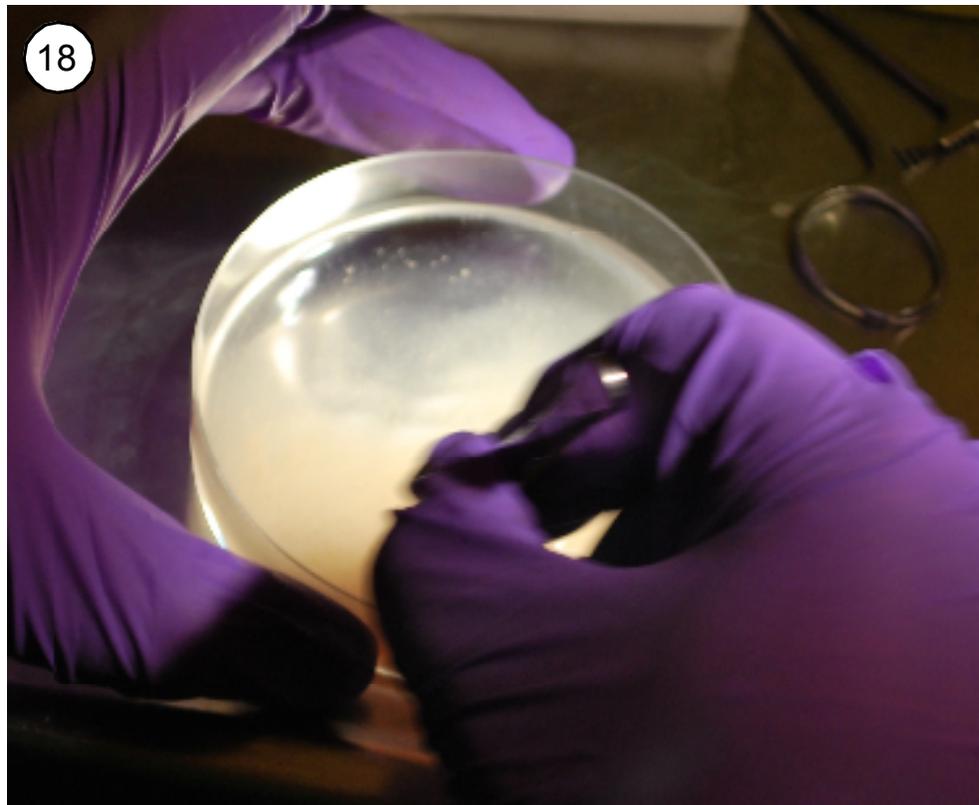
Cutting out the perfused liver

13. Make sure there are no bubbles entering into the system!
14. When the liver is digested, (bubbly appearance, very light color, separation of Glisson's capsule from parenchyma), remove the canula.
15. (See previous page for illustration of step 15) Remove the liver carefully, by cutting the ligaments with small scissors. Take great care not to rupture the esophagus!
16. Very carefully remove the gall bladder.

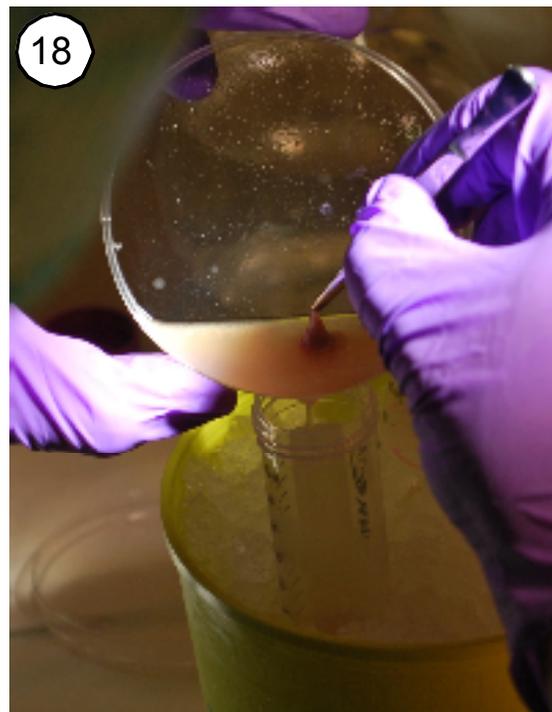


Removing Glisson's capsule and releasing dispersed cells

17. (See picture above) Place the liver on the petri dish (with some perfusion or preservation buffer in it), and remove the Glisson's capsule.
18. (See next page for 2 pictures illustration of step 18). Shake the liver gently to release the cells. When the buffer seems saturated with cells, pour the cell suspension into a 50 ml tube and add more ice-cold preservation buffer.
19. Repeat this process as long as necessary.
20. Top the cell suspension with preservation buffer.
21. Keep the cell suspension at 4°C or on ice.
22. Wash the system with 50 ml of water and then fill with 70% ethanol to leave it until next liver perfusion.



Shake liver gently to release dispersed cells into the buffer



Pouring dispersed cells into tube

PROCEDURE FOR CELL ISOLATION AND CULTURE:

1. Very gently mix cell suspension.
2. Centrifuge: 54 x g for 2 min at 4°C. Set acceleration/deceleration to max.
3. Collect the supernatant by pouring out or by pipette.
4. Centrifuge: 54 x g for 2 min at 4°C. Set acceleration/deceleration to max.
5. Gently collect the supernatant by pipette.
6. Centrifuge: 1350 x g for 10 min at 4°C. Set acceleration/deceleration to max.
7. Resuspend pellet in 10 ml preservation buffer. Load on top of 25/50% Percoll gradient (see buffer preparation below).
8. Centrifuge: 1350 x g for 30 min at 4°C. Set acceleration/deceleration to minimum.
9. Collect the non-parenchymal cells (NPC) from the interface between the two density cushions of 25% with 50% Percoll with a 10 ml pipette. (The Percoll solution can be changed to 23%, which gives more NPC but also more stellate cells. When the animal is very fat use 45% instead of 50% Percoll to obtain less hepatocytes.)
10. Resuspend collected NPC in 50 ml preservation buffer.
11. Centrifuge 1350 x g for 10 min at 4°C. Set acceleration/deceleration to max.
12. Resuspend pellet in 10 ml RPMI culture medium.
13. Count the cells.
14. Remove the Kupffer cells by selective adherence: incubate cell suspension in non-treated plastic Petri dish (must be Falcon) for 8 min (incubator, 37°C).
15. Shake the plate and transfer the medium to another Petri dish (Petri dish-2).
16. Wash Petri dish-1 with 5 ml RPMI medium and add to Petri dish-2.
17. Incubate another 8 min (incubator, 37°C). Transfer the medium to 50 ml Falcon tube, wash Petri dish-2 with 10 ml medium and add to the same 50ml Falcon tube. Then add preservation buffer to 50 ml.
18. Centrifuge: 1350 x g for 10 min at 4°C. Set acceleration/deceleration to max.
19. Resuspend pellet in 2.7 ml of RPMI.
Count the cells in a Bürker chamber/Hemocytometer
20. Seed the cells on collagen-coated cell-culture plastic dish. Small dish (9.6 cm²), up to 3.5-4 million cells in 1.4 ml; Intermediate dish (19.2 cm²) up to 7 million cells in 2.7ml; Big dish (56.7 cm²) up to 21 million cells in 7.6 ml; 24 well plate: 1 million cells per well.

Coating of dishes with collagen

- Add 50 µl of freshly prepared Vitrogen stock (2.9 mg/ml) in 50 ml of PBS → 0.0029 mg/ml or 0.00029% collagen.
 - Add 300µl of above mixture to each well of 24 well cell culture plate; *or* 1.4 ml to small dish; *or* 2.7 ml to intermediate dish.
 - Incubate 20 min at room temperature.
 - Discard collagen solution from the plate. If cells are not ready to be seeded right away, then supply each well (or dish) with PBS and store at room temperature until cells are ready.
21. Incubate LSECs in dish for 35 min (incubator, 37°C). (Note that LSECs from different mouse strains may need longer or shorter incubation time to attach and spread optimally.)
 22. Wash with cold PBS (pipette).
 23. If parenchymal cells are still present in the culture: wash intensively with a Pasteur pipette. LSECs stay attached during such this flushing; parenchymal cells do not.
 24. Cells are now ready for RNA or protein isolation.

25. For endocytosis studies or for microscopy incubate the cells for another 1h in RPMI in order for them to spread optimally.

BUFFERS FOR PERFUSION

Perfusion buffer concentrate (PBC):

Dissolve NaCl (103.75 g), KCl (6.25 g) and Hepes (28.70 g) in H₂O (350 ml) while stirring.

When all has been dissolved add 1M NaOH (75 ml).

Add H₂O to a total volume of 500 ml.

Filter the solution (0.45 μ m pores) and divide in tubes (50 ml); freeze in portions of 40 ml.

Perfusion buffer:

Dilute 40 ml of PBC by milliQ water to a total volume 1 litre.

Preservation buffer:

Add 2.5 g of BSA to 250 ml of perfusion buffer. Let it dissolve. Keep at +4°C or on ice.

Dissociation buffer:

Add 0.5ml of 476 mM CaCl₂ to 49.5ml of perfusion buffer to obtain a final concentration of 4.76 mM CaCl₂. Add 1.5 mg of freshly defrosted stock of Liberase TM (Roche) just before perfusion start.

PERCOLL for one (25%/50%) gradient		
Stock Percoll	ml	13.5
10X PBS	ml	1.5
Result "100%" Percoll	ml	15
50% Percoll		
100% Percoll	ml	10
1X PBS	ml	10
Result - 50% Percoll	ml	20
25% Percoll		
100% Percoll	ml	5
1X PBS	ml	15
Result - 25% Percoll	ml	20

FREQUENTLY ASKED QUESTIONS

1. * Is it really necessary to remove the Glisson's capsule from the liver? Is there a special technique to remove it?

It is not necessary to remove absolutely all of the Glisson's capsule; what is meant is that Glisson's capsule has be ruptured and pulled off by means of tweezers so that the dissociated liver cells can be gently shaken out into the buffer.

2. * During the cell preparation, the protocol states that you resuspend the pellet in RPMI culture medium. What type of RPMI do you use, and do you supplement the medium with anything?

We use "normal RPMI" that we purchase from PAA (product # E15-039, which is without L-glutamine) [see also

http://www.paa.com/products/cell_culture_products/media/classical_media/rpmi1640/rpmi_1640_formulation.html. Just before use we supplement the RPMI with L-glutamine/penicillin/streptomycin (product # P11-013). [see also http://www.paa.com/cell_culture_products/reagents/aminoacids_vitamins/l_glutamine_penstrep.html]

NB: The presence of serum in these cultures may markedly affect morphology and scavenger function of the cells negatively.

3. * The Kupffer cells are removed from the cell suspension by selective adherence in non-treated plastic Petri dishes. What do you mean by "non-treated"?

"Non-treated dishes" means ordinary tissue culture dishes (Falcon gives best result) that have not been precoated with proteins.

4. * What are your approximate yields of KC and LSEC from 1 Balb/C mouse? Is the procedure mostly carried out on more than one mouse?

Yields are variable, and depend somewhat on the gender (more cells from males), age (relative amounts of KCs and stellate cells increase somewhat with age) and strain (higher yield from Balb/c than from C57BL/6). The usual yield of KC+LSEC per C57BL/6 mouse (3-6 months old, 25-30g body mass) is 5-10 million. From Balb/C mice you can expect 50-75% higher yield. Approximate KC to LSEC ratio is 1:3.5 (difficult to give a precise estimate unless you use pronase perfusion with subsequent centrifugal elutriation).

Depending on the need of cells and the type of experiment we include one or up to 3-4 mice in one preparation procedure.

5. * What are the yields and purities of the final cell populations?

Purity in the final primary cultures after first wash depends on incubation time before washing. Purity of LSEC is within 90-95% (as judged by fenestration assessed by SEM, and positive staining for the LSEC specific stabilin 2). The remaining 5-10% of cells in these cultures represent Kupffer cells/monocytes and stellate cells.

6. * How do you deal with the collagenase?

We reconstitute the small pack size of Liberase Blenzyme 3 (1X) in 1ml H₂O (NOT in 2ml). It gives us the same concentration of enzyme as the big pack size of Liberase Blenzyme 3 (10X) reconstituted in 10ml. As soon as you reconstitute it in 2ml you should double the volume of enzyme stock, which you add to 50 mL of perfusion buffer (i.e. 0.572ml).

Important! Thaw the Liberase stock and add it to the perfusion buffer just before perfusion.

7. * Do you also filter the preservation buffer with the BSA (0.45 μm)?

No, but it would not hurt if you do.

8.* Step 20 in the procedure (*top the cell suspension with preservation buffer*). You mean with this "add a layer of preservation buffer on top of the cells in the Falcon"? Or do you have to add preservation buffer until the Falcon tube is completely filled?

The meaning is: add preservation buffer until the Falcon tube is completely filled (if it is not already full after procedure). Mix it gently. And you should also mix it gently again just before centrifugation.

9.* How much KC do you seed /petri dish? Are the KC also seeded in the 35-3004 petri dishes from Falcon (60 mm)?

We are normally removing KC by adhesion to plastic. Therefore it is difficult to say how much KC we seed. Normally we seed the non-parenchymal cell suspension from one mouse in one big Petri dish (56.7 cm²), steps 14-16.

10.* How long can you keep the KC in culture?

Normally we do not keep KC for long - if at all. [Our main line of research includes studies on LSECs; thus we normally just want to get rid of the KCs.] However, you should be able to keep them for 48 hours or even longer if you add growth factors or serum to the medium.

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