**Detailed Protocol for Rat OPC Isolation (MACS Kit)**

**1 day prior to isolation**:

1. PDL coat 6 cm TC dish for 1.5 – 2 hours. Wash 3x with dH20, allow to dry. Parafilm and store overnight at 4°.
2. Make 2 x 40 ml MACS Buffer from MACS Stock:
	1. To make MACS Stock, make 1 L DPBS from powder (Invitrogen 21600-010). Add 1 g/L glucose to make DPBS-G. Add 4 ml phenol red as a pH indicator, then 1 ml 0.5M EDTA (Sigma E-7889)(0.5mM final). Filter and store at RT
	2. To make 40 ml MACS Buffer, take 36 ml stock solution and add 4 ml 5% BSA solution (Sigma A4161) and 100 ul aliquot insulin (5 ug/ml final). Filter through a 0.22 um PVDF syringe filter, parafilm, and store at 4°.

**Isolation Day**:

1. Place PDL-coated dish in hood with lid partially off to dry completely. Add Laminin to coat for at least three hours prior to cell plating.
2. Make 10 ml 90% Percoll (9 ml) + 10% 10x HBSS w/o Ca2+ & Mg2+ (1 ml). Parafilm and store at 4°.
3. Perfuse two adult rats with cold 1xPBS. Remove the spinal cord using the “Cord Squirt Method” into fresh HBSS w/o Ca2+ & Mg2+. Remove nerve roots and dura, then transfer to fresh HBSS w/o Ca2+ & Mg2+.
	1. Cord Squirt Method – After perfusion, cut the fur along the back of the animal and pull to the sides to effectively skin it. Remove the internal organs; you can do this quickly by holding the animal vertically with the head up and cutting behind the organs to remove connections and allow the organs to spill out freely. Use bone cutting or other durable scissors to cut between the shoulder blade and rib cage, then continue cutting down along the rib cage to the hip. Turn scissors perpendicular to the spinal column and cut just above the hips to remove the lower portion of the animal. Similarly, cut near the shoulder blades to remove the head and upper portion of the animal. You should now have the spinal column plus some bone and tissue, but not much else. Use a 60 ml syringe loaded with sterile DPBS and a yellow pipet tip (we super-glued our pipet tip and syringe together so we could re-use it for multiple preps) to squirt the cord out of the column. Be sure to insert the tip into the vertebral foramen at the rostral end of the spinal column. It will take a fair amount of force to squirt the cord out, but it should come out the caudal end of the spinal column in one piece. Sometimes it will split into two hemispheres during the removal process, but this does not adversely affect OPC viability or yield.
4. Prepare 3x volume of Enzyme mix 1 (5.7 ml Solution 2 + BME, 150 ul Solution 1). Vortex, then preheat in 37° water bath for 5 minutes before use.
5. In the hood, dice the cord in 1 ml fresh HBSS w/o Ca2+ & Mg2+. Cut the tip off of a 1 ml plastic barrier pipette tip (red box), and use this to transfer cord pieces to a 15 ml conical tube (cord pieces will stick to regular 5 ml pipettes!). Rinse the plate several times to get all the tissue, then spin 2 min at 300g.
6. During the spin, add required amount of rOPC + 1X growth factors medium (always add a couple of mls extra per dish) to a tissue culture dish and place in the CO2 incubator.
7. When the spin is finished, aspirate supernatant carefully. Add the pre-heated Enzyme Mix 1 to the cells and triturate gently once or twice, avoiding air bubbles. Incubate 15 min in the 37° water bath, inverting the tube several times every 5 min to resuspend settled cells.
8. During incubation, prepare Bead 1 Mix (180 ul MACS Buffer + 20 ul Rat anti-mouse IgM beads) and O4 mix (360 ul MACS Buffer + 40 ul O4 hybridoma). Store on ice next to hood.
9. Also during incubation, prepare 3x volume of Enzyme Mix 2 (30 ul Solution 4 + 60 ul Solution 3). Add Enzyme Mix 2 to the tube already containing cells and Enzyme Mix 1. Invert gently to mix. **Do Not Vortex!**
10. Dissociate tissue mechanically using the widest fire-polished pipette by triturating ~15 times slowly. **Do not form air bubbles!**
11. Incubate in water bath for 10 min, inverting several times every 5 min.
12. Dissociate tissue mechanically using the other two fire-polished pipettes in decreasing diameter. Triturate gently 15-20 times with each pipette, or as long as tissue pieces are still visible, being careful to avoid air bubbles.
13. Incubate in water bath for 10 min, inverting several times every 5 min. During incubation, turn on the Sorvall centrifuge to start it cooling down.
14. Pre-wet a **70** micron strainer (top shelf above Jason’s bench) with 5 ml DPBS into a 50 ml conical tube. Aspirate DPBS from tube. **Slowly pour** the single-cell suspension into the strainer, wash the 15 ml tube that had the cells with 10 ml of HBSS **with** Ca and Mg, and add this to the strainer. Discard cell strainer and centrifuge at 300 x g for 10 min at RT.
15. Gently aspirate supernatant, being cautious not to disturb the loose cell pellet. If necessary, leave a small volume of supernatant behind to avoid disturbing cells. Repeat wash with 10 ml HBSS **with** Ca and Mg (triturate gently to resuspend cell pellet). Spin 300 x g for 10 min at RT.
16. Aspirate supernatant and resuspend cells in MACS Buffer to a total volume of 8.5 ml, including tissue. To do this, I usually resuspend in about 7 or 7.5 ml MACS Buffer, transfer to the ultracentrifuge tube, then add whatever volume I need to make 8.5 total ml. Add 5.7 ml 90% Percoll gradient to the ultracentrifuge tube. Mix gently but thoroughly by trituration. Weigh the tube and balance it exactly with a 2nd ultracentrifuge tube plus water. Program the Sorvall to Rotor SS-34 (Code 05), Speed = 15800 rpm, Time = 15 min, Temp = 4°. For non-KSCIRC protocol users, match your rotor and centrifuge to the speed, time, and temp listed above.
17. Remove the ultracentrifuge tube VERY CAREFULLY! Do not disturb the myelin layer! Back in the hood, aspirate the myelin laver very carefully. Remove the remaining cell suspension layers to a sterile 50 ml tube. Add an equal volume of MACS Buffer and spin 10 min at **1200g** (not rpm!). During spin, use a 5 ml pipette to scrape off and discard the gelatinous residue off the inside of the ultracentrifuge tube so it doesn’t congeal.
18. Aspirate the supernatant and wash again with a few ml MACS Buffer. Spin 5 min at **1200 rpm** (not g!)
19. Aspirate the supernatant and resuspend in 5 ml MACS Buffer. Remove a small aliquot for a cell count (I usually dilute 20 ul cells in 20 ul Trypan Blue for a 1:2 dilution). Spin cells for 5 min at 1200 rpm. Count total cells during the spin.
20. Gently aspirate the supernatant. Add the diluted MACS beads prepared earlier (180 µl MACS Buffer + 20 µl Beads). Resuspend the pellet very gently, avoiding air bubbles. Incubate 15 min at 4° with gentle shaking, mix by tapping every 5 min. Resuspend in 5mls MACS buffer. If you notice any floaters that can’t be resuspended, strain the solution though a pre-wet 40µ strainer, and wash the strainer with an additional 4 ml MACS buffer.
21. During the incubation, make up second MACS Beads mix based on cell count. For 1 x 107 cells, mix 20 µl MACS Beads with 180 µl MACS Buffer. Scale up both volumes as necessary based on cell count. Ex: 3 x 107 cells = 540 µl Buffer + 60 µl Beads.
22. Following 15 min incubation, spin cells 5 min @1200rpm. During the spin, set up and equilibrate a MACS column with 0.5ml MACS buffer. If possible, position a 15 ml tube at an angle below the column to allow the solution that flows through to gently slide down the inside of the tube (this minimizes introduction of bubbles).
23. Resuspend the pellet in 0.5ml MACS buffer and add to column. Wash 4x 0.5ml MACS buffer. Collect the initial flow through and all washes in single 15ml tube. Spin this 5 min @1200rpm.
24. Resuspend in 400µl diluted O4 antibody as before. Incubate 5 min at 4° with gentle shaking, mix once by tapping. Wash 2x 5ml MACS (for a wash, resuspend in 5 ml MACS, spin down 5 min at 1200 rpm, then aspirate MACS. Do this twice).
25. Resuspend in second MACS beads mix. Incubate 15 min at 4° with gentle shaking, mix by tapping every 5 min. Resuspend in 5ml MACS buffer. If you notice any floaters that can’t be resuspended, strain the solution though a pre-wet 40µ strainer, and wash the strainer with an additional 4mls MACS buffer.
26. Spin 5 min @1200rpm. During the spin, set up and equilibrate a MACS column with 0.5ml MACS buffer.
27. Resuspend the pellet in 0.5ml MACS buffer and add to column. Wash 4x 0.5ml MACS buffer.

During 2nd or 3rd wash, remove Laminin from dish and wash 1x with DPBS. Leave lid partially or completely off to dry.

\*\*\*It is very important that the dish is completely dry before adding cells! The plate is ready when the Laminin coating dries to a uniformly opaque film.

1. Elute the bound cells in 1ml MACS buffer into a fresh 15 ml tube. Angle the column so that the elutant slides gently down the inside of the tube. Bubbles will be introduced during this step, but try to minimize their contact with the cell suspension at the bottom of the tube.
2. **Equilibrate a fresh column**, and then add the eluted cells. Wash 4x 0.5ml MACS buffer. Elute into a fresh 15 ml tube in 1ml equilibrated mOPC-A medium, again angling the column down the inside of the tube. Add a second ml of medium to the cell suspension and mix thoroughly but gently (minimize bubbles as usual).
3. Count cells, and plate for expansion and staining. I usually dilute 20 ul cell suspension in 20 ul Trypan Blue for this count. Plate in 6 cm dish in 3 ml rOPC + 1x GF medium.

**Post isolation**:

1. First Day: Pre-equilibrate 5 ml Rat OPC Medium in incubator for 20 – 30 minutes.
2. Aspirate all medium from Rat OPC plate and replace with fresh medium.
3. Subsequent feedings should be ½ medium changes every other day for about 7-10 days. Cells can then be passaged and expanded or frozen in 10% DMSO for further experiments.

**Passaging**:

1. PDL/Laminin coat appropriate plates before passaging. Also pre-equilibrate enough medium for all plates, plus an additional 5 ml or so per plate for washes.
2. Aspirate all medium from Rat OPC Plate(s). Rinse plate 1 x with DPBS, aspirate, then add appropriate volume 0.05% Trypsin (6 cm dish = 3 ml, 10 cm dish = 5 ml).
3. Place plate in incubator for 3 minutes. Back in the hood, remove the cell suspension to a 15 ml conical tube, rinse the plate with 4 ml Rat OPC Medium and add to tube, then spin 5 minutes at 1200 rpm.
4. Count and plate cells as usual.

**PDL/Laminin Coating Plastic ware:**

PDL:

100X PDL Stock (1mg/ml) – Add 5ml dH2O to a 5mg bottle of Poly-D-Lysine (Sigma P6407) in the hood. Leave at 4°C overnight, making sure the bottle stays upright. The next day gently mix the contents by swirling. Make 0.5ml aliquots IN CRYOVIALS. Store at -80°C. Each aliquot can be diluted in 50ml dH2O to make 1x PDL (5 µg/ml)

Coating: Add PDL to plates/dishes. Incubate in hood for 1-3 hours. Aspirate the PDL, wash 3x dH20. Dry, with the lids partially open, at the back of the hood for 20-30 minutes. The coated plastic ware can be Laminin coated immediately, or stored at 4°C for up to 1 week.

Laminin:

Thaw a 1mg vial of commercial Laminin (Sigma L2020-1mg). Add to 50ml DPBS to make a 20µg/ml working stock.

Add appropriate amount to PDL coated plates/dishes. Incubate at RT in hood for 3 hours or longer. The laminin can be reused once, so be careful to keep it sterile. Add the used Laminin solution to a fresh 50 ml conical if this is its first use; discard if it has already been used. Rinse the plastic ware 1x DPBS and dry dishes with lids partially open in the hood. Dishes are sufficiently dry when there is a visible opaque film covering the surface.

I prefer Laminin coating dishes on the day of use.

**Rat OPC Medium**:

DMEM/F12 (w/o HEPES) + 2.1 g/L NaHCO3 + 1xN2 + 1xB27 + 1% P/S + 20 ng/ml FGF2 + 10 ng/ml PDGFaa + 5 µg/ml Insulin + 0.1% BSA.

Rat OPC medium is very similar to the mouse OPC medium (mOPC-A) we make. Thus, you can make 100 ml of rat OPC medium from the mouse OPC medium (1x Growth Factors!)

mOPC-A (1x G.F.) 100 ml

5% BSA 2 ml (0.1%)

Insulin 250 µl of aliquoted insulin (5 µg/ml final)