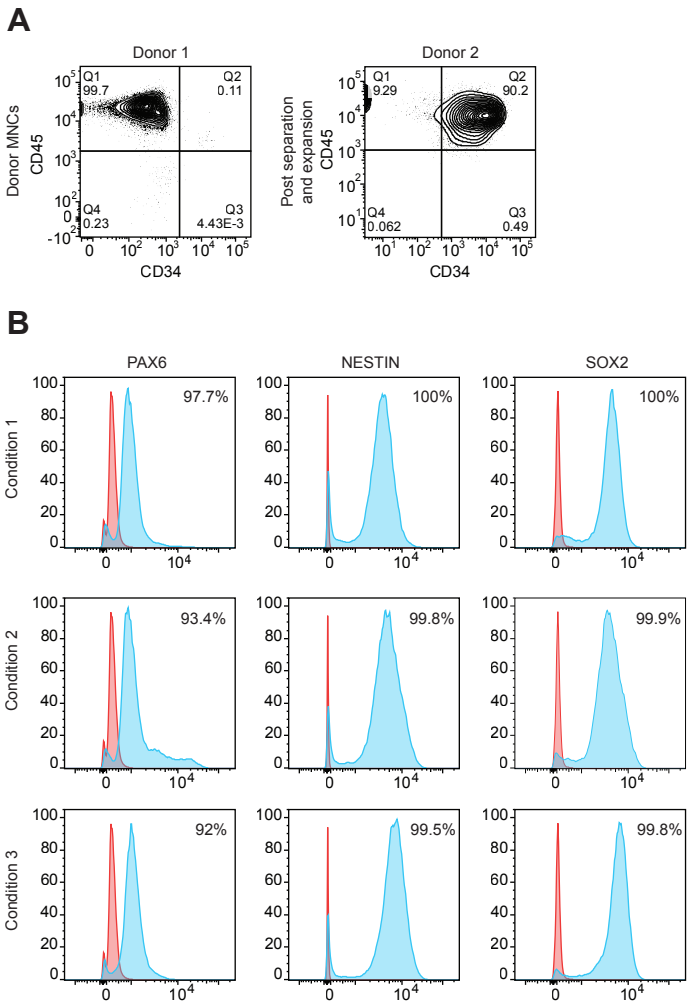
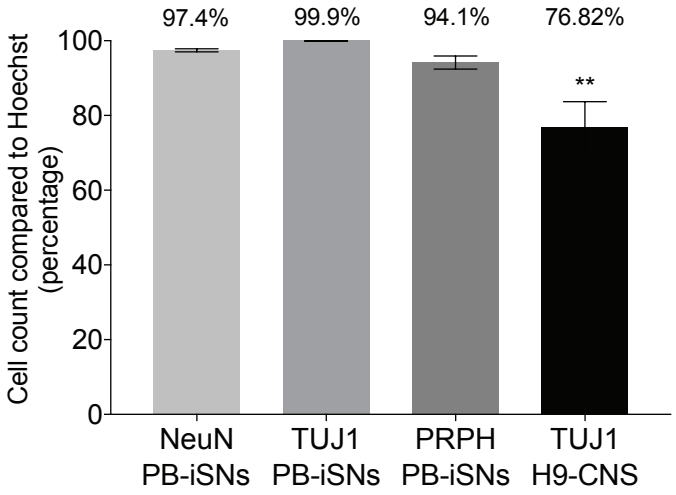
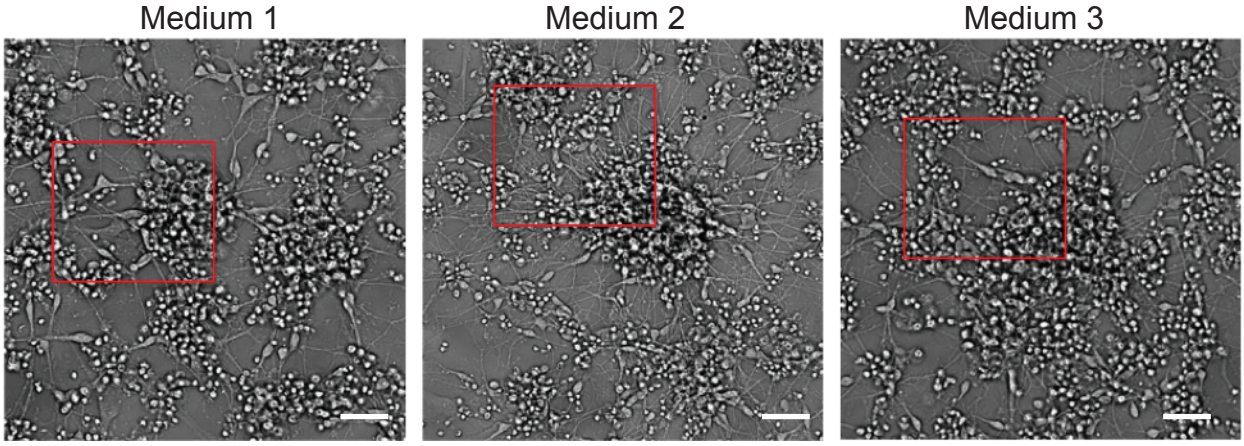

A
Donor 1
Donor 2
Donor MNCs CD45
Post separation and expansion CD45
CD34
Q1 99.7
Q2 0.11
Q4 0.23
Q3 4.43E-3
Q1 9.29
Q2 90.2
Q4 0.062
Q3 0.49
B
PAX6
NESTIN
SOX2
Condition 1
Condition 2
Condition 3
97.7%
100%
100%
93.4%
99.8%
99.9%
92%
99.5%
99.8%

A

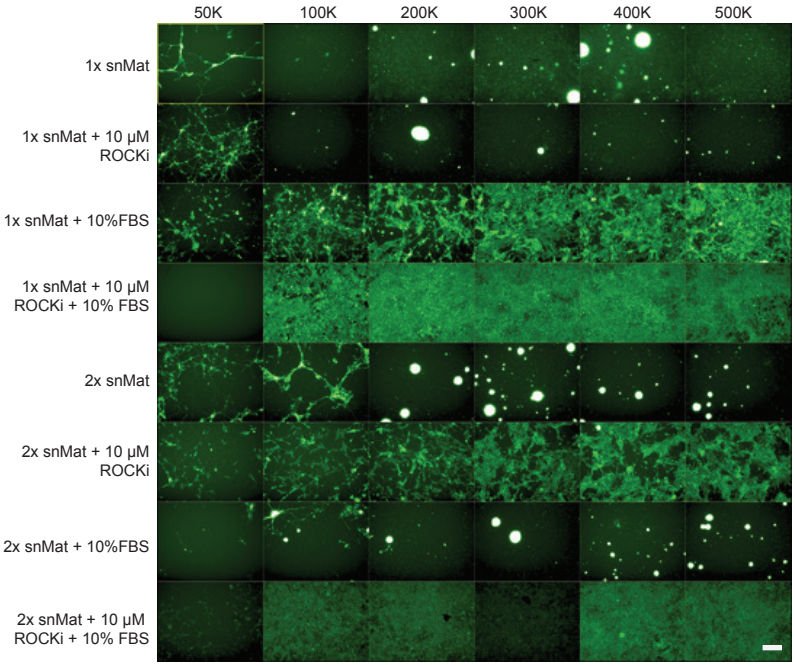

97.4%
99.9%
94.1%
76.82%
**
Cell count compared to Hoechst (percentage)
100
80
60
40
20
0
NeuN PB-iSNs
TUJ1 PB-iSNs
PRPH PB-iSNs
TUJ1 H9-CNS

B

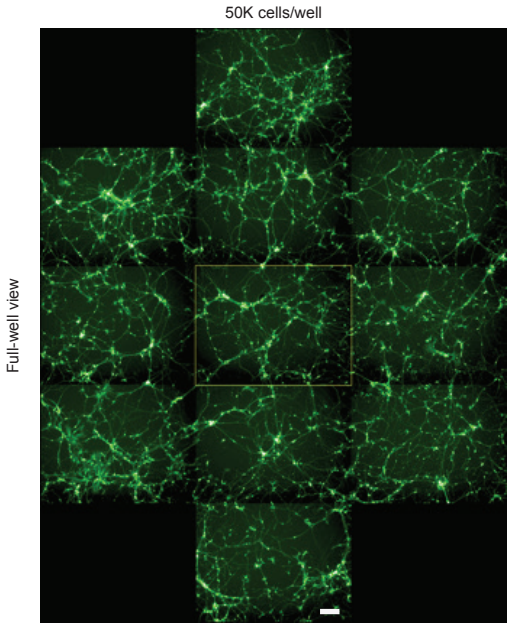

Medium 1
Medium 2
Medium 3

M.Bhatia et al, Supporting Information Figure 3

A

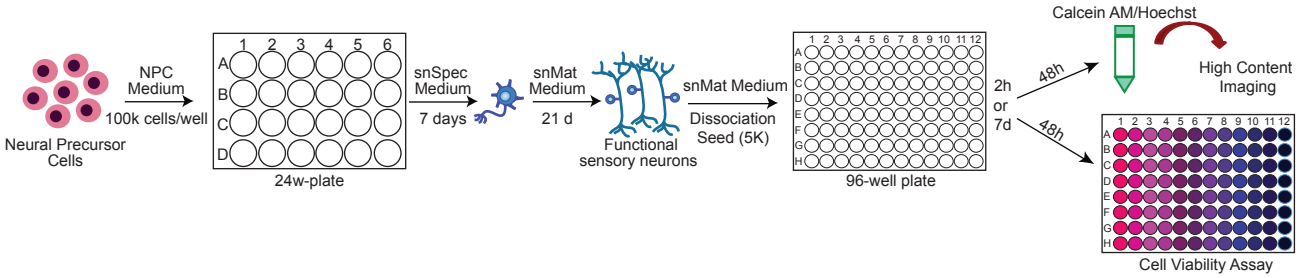


B

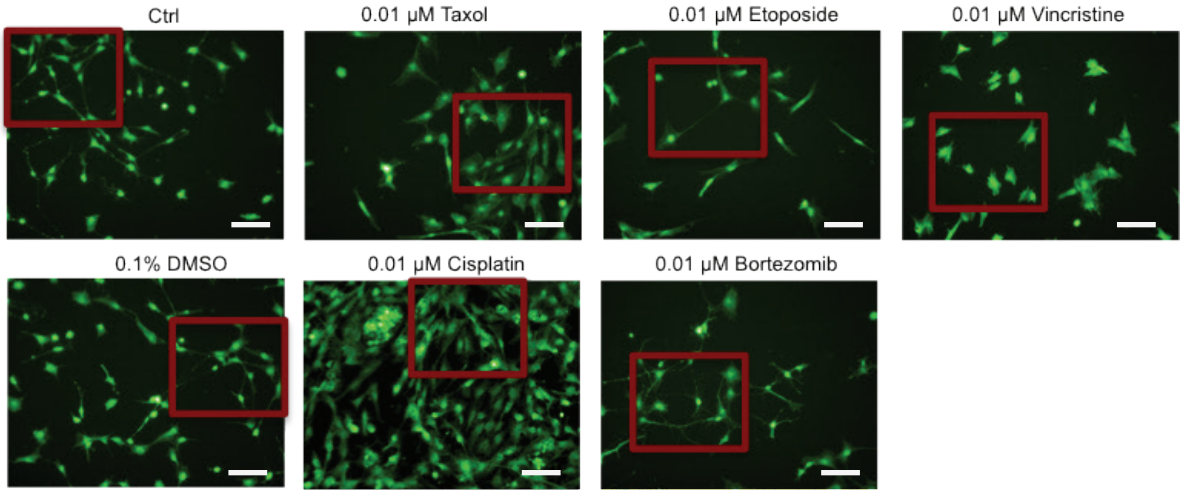


M.Bhatia et al, Supporting Information Figure 4

A



B



Protocol for thawing primary peripheral blood mononuclear cells

Reagents and initial setup:

- PEF (3% FBS in PBS + 1mM EDTA) warmed up to 37°C (Filter sterilize).
- FBS (100%) warmed up to 37°C.
- 100-1000ul pipette-man (set to 1ml) and pipette-tips.
- 25ml serological pipette.
- Pipette gun.
- 50ml falcon tube.
- 37°C water-bath.
- PBS-EDTA buffer: PBS containing 0.5% BSA and 2mM EDTA (Filter sterilize).
- This procedure should be done as quickly as possible.
    - Set everything (reagents) up by bring all the necessary reagents into in the biosafety cabinet (BSC) after surface sterilization by wiping with 70% ethanol
    - Warm up the media in the 37°C water-bath outside the BSC.
    - Dial the pipettes to minimize any delay in the thaw procedure.
    -

Procedure:

- Warm cell culture medium in a 37°C water bath.
- Takeout the vial containing peripheral blood MNCs from liquid nitrogen tank.
- Wipe the outside of the vial of cells with 70% ethanol.
- In the BSC, twist the cap (of the vial) a quarter-turn to relieve the internal pressure and t hen re-tighten.
- Immediately thaw the cells in a 37°C water-bath by gently shaking/swirling the vial (in the 37°C water bath). Constantly inspect the tube until there is just a small bit of ice left in the vial.
- Quickly transfer the vial into a BSC and carefully wipe the vial with 70% ethanol and/or with freshly prepared “activated Clidox “(1:18:1 – Clidox base: Water: Clidox Activator) and open the vial.
- Gently but quickly transfer the cryopreserved cells into an empty 50ml falcon tube the P1000 pipettor.
- Add 1ml of 37°C FBS directly on the cells in the falcon tube drop-wise while gently swirling the tube.
- Add up to 29ml of 37°C PEF onto the cells drop-wise while swirling the tube. Rinse the leftover cells in the freeze-vial with 1ml PEF and add to cells.
- Centrifuge the cell suspension at 1200rpm for 10 minutes.
- After centrifugation, aspirate the supernatant without disturbing the cell pellet.
- Gently resuspend the cell pellet in 5-6 ml of PBS-EDTA buffer for further processing of enrichment of CD34+ve cells medium with a pipettor. If there are any clumps, break them with a pipette tip. Remove the cell clumps if they cannot be resuspended by passing through cell strainers. Do not use DNAse I if the cells are going to be used for RNA or DNA extraction.
- Take small aliquot and perform cell-counting assay by Trypan blue. Determine cell number using hemocytometer or cell countess.
- Take 800-900K cells for Flow analysis to measure percentage of CD34+ cells in the sample.
- With resuspended PB MNCs in 50 ml conical tube, centrifuge cell suspension at 1200rpm for 10 minutes. Aspirate the supernatant completely.
- Resuspend cell pellet in 300 µL of PBS-EDTA buffer for up to 10^8 total cells.
  (If you have more cells; for example $2X10^8$ cells, resuspend in 600 ul)

Enrichment of CD34+ve cells

Note: Lineage depletion will reduce yield in favor of purity.
Enrichment of CD34+ve cells was performed using (Miltenyi Biotech) CD34 MicroBead Kit (human) (Cat# 130-046-702)

Reagents and initial setup:

- CD34 MicroBeads, human: MicroBeads conjugated to monoclonal mouse anti-human CD34 antibodies (isotype: mouse IgG1) -2 ml suspension.
- FcR Blocking Reagent, human: Human IgG.- 2ml suspension.
- PBS-EDTA Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Keep buffer cold (2−8 °C).
- MACS® LS Column (Cat# 130-042-401).

Procedure:

- Add 100 µL of FcR Blocking Reagent (suspension) for 10^8 total cells that are present in the 50 ml tube (in PBS-EDTA buffer); as per the kit we are suggested to add 100 ul of the FCR blocking reagent for each 10^8 total cells.
- Add 100 µL of CD34 MicroBeads (suspension) to the tube containing 10^8 total cells (PBMNCs). (Note: For more cells, for example $2X10^8$ cells, add 200ul of blocking and CD34 microbeads).
- Mix well and incubate for 30 minutes in the refrigerator (2−8°C).
- (Note: During this incubation, swirl the tube to mix the cells and antibody after every 10 mins.)
- Wash cells by adding 5−10 mL of buffer for up to 10^8 cells and centrifuge at 1200rpm for 10 minutes.
- Aspirate supernatant completely.
- Resuspend up to 10^8 cells in 3mL of PBS-EDTA buffer.
- (Note: For higher cell numbers, scale up buffer volume accordingly. E.g., for $2X10^{10}$ cells resuspend in 5 ml of the PBS-EDTA buffer)
- Proceed to magnetic separation.
  (Note: Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD34+ cells. Place MACS LS column in the magnetic field of a suitable MACS Separator.

Column	Max. number of labeled ells	Max. number of total cells
MS	10^7	$2x10^8$
LS	10^8	2x19
XS	10^9	$2x10^{10}$

- Prepare column by rinsing with 3 ml of PBS-EDTA buffer.
- Always wait until the column reservoir is empty before proceeding to the next step.
- Apply cell suspension onto the column using P1000 pipette.
- Collect flow-through containing unlabeled cells (the cells that do not express CD34 antigen/CD34-ve cells).
  Wash column with 3 ml of PBS-EDTA buffer. Repeat this step 3 times. (Note: Perform washing steps by adding buffer aliquots only when the column reservoir is empty)
- Collect unlabeled cells (CD34-ve cells) that pass through and combine with the flow-through from wash steps. Remove column from the magnetic separator and place it on a suitable collection tube.

- To elute the CD34+ cells, pipette the 3 ml of PBS-EDTA buffer onto the column.
- Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. (The plunger is provided with the kit. The plunger should be used only at the final step of elution, not any other steps)
  (E.g., before column separation the expected number of CD34+ cells were 460,000 with 0.20% occurrence. However, after column separation 226,500 CD34+ were collected which reduced the yield about 50% but increased the purity from 0.20% to 15.1%)
- Take a small aliquot (20µl) for cell counts (hemocytometer or countess)
- Take 50-100K (depending on the cell count) for flow to measure CD34+ cells after column separation.
- Centrifuge the cells at 1200rpm for 10 minutes. Note: higher rpm reduces viability.
- Remove the supernatant and resuspend the cells in StemSpan medium containing hSCF, Flt3L and TPO.

Culture of CD34+ve cells

Reagents and initial setup:

- StemSpan medium containing hSCF, Flt3L and TPO (warmed at 37ºC water bath).
- Non-treated tissue culture plates

Procedure:

- After magnetic separation, spinning down CD34+cells and resuspend in StemSpan medium. (Note: seeding density- if isolated <100K CD34+ cells, plate them in 24 well plate (non-treated tissue culture plate) at a density of 100K cells/ml culture medium. If the number of CD34+ cells are higher than 200K cells, plate them in 6 well plate (non-treated tissue culture plate) at density of 100K cells/ml.)
- After plating cells, rock the plate so cells distribute evenly in the plate and incubate at 37ºC.
- Replace the culture medium with fresh medium every other day as following. Collect the cell suspension and spin down at 1200rpm at 10 mins at room temperature. Aspirate the culture medium without disturbing the pellet.
  (Note: If pellet is disturbed at any stage after centrifugation repeat the centrifugation step.)
- Resuspend the cells with the fresh medium and plate the cells in same plate/well from which the cells suspension was taken.
- Culture cells for 3 days for expansion in non-treated tissue culture plate.
- After 3 days expansion, count the cells by countess.
- Take a small aliquot of cells for Flow cytometry analysis for CD34 expression.
- (Note: after 3 days you will notice 2.5 to 3 fold increased in number of CD34+ cells as well as purity of CD34+ cells from the initial seeded density of CD34+ cells.
- Cells are ready for further processing.

Protocol for viral transduction of CD34^{+ve} cells with Lenti-OCT4 viral particles

Reagents and initial setup:

- Culture medium for CD34^{+ve} cells.
- High titre Lenti-OCT4 virus.
- Retronectin
- PBS- (sterile)
- Polybrene (10 mg/ml-stock)
- Reprogramming medium: 90% DMEM/F12+10% KOSR+1% NEAA+0.5% L-Glut
- (*** BME (stock 1:10 diluted) - 7ul for 100ml final volume medium – calculate the amount of BME for different volume of final medium)
- Neural induction medium: DMEM/F12, 1XN2, 1XB27, LDN (100nM), SB (10 uM), CHIR (3 uM), BFGF (20 ng/ml) and EGF (20 ng/ml),
- BSL2+ facility
- Training and clearance to work in a BSL2+ facility

1. **Prepare and store the Lenti-OCT4 virus as per the lentiviral production SOP in the BSL2+ facility.**
2. **Book BSL 2+ hood/BSC for Lenti-OCT4 viral transductions**
3. **By following BSL2+ SOPs/protocols, open the BSL2+ hood/BSC2 hr prior to the Lenti-OCT4 transductions.**

Procedure:

- Follow the protocol iNC01, iNC02 and iNC03 to isolate CD34+ve cells from peripheral blood.
- Prior to the Lenti-OCT4 infections, coat 24 well-plate (non-TC treated) (required number wells considering 100K cells/well/infection) with Retronectin (40 ug/ml). Coating should be done 2 hr at RT and then over-night at 4°C.
    - Retronectin coating protocol:
        1. Prior to coating, prepare a Retronectin solution by taking required amount of Retronectin and dilute in PBS to make a final concentration of 40 ug/ml (stock 500 ug/ml).
        2. To avoid loss of Retronectin fragment, do not filter sterilize Retronectin solution diluted with PBS.
        3. Dispense 300ul/well-24 well plate of Retronectin solution (40 ug/ml) into each well and allow the plate to stand for 2 hours at room temperature and at 4°C overnight. (Dispense 0.5 ml into each well of a 12-well plate or 2 ml into each well of a 6-well plate). Note: Non-treated/Non-adherent, cell culture-grade tissue culture plates or dishes should be used.
        4. Next day, remove Retronectin Solution and wash the plate once with PBS. After removing the PBS the plate is ready to use.
- After 3 days of culturing, count the cells with hemocytometer or cell countess by taking 20 ul of cell suspension and determine the cell number.
  (Note: Expected density would be 2.5 to 3 times to the starting number of cells. For example if 100K cells were seeded and after expansion one could expect 250K to 300K cells.)
- Collect the cell suspension into a 15 ml falcon tube. Wash the cell culture well with sterile PBS and collect it into the same falcon tube.
- Centrifuge cells at 1200 rpm for 10 min.

- After the centrifugation, carefully remove the supernatant and retain the cell pellet.
- Resuspend the cells in $CD34^{+ve}$ cell culture medium to achieve a final cell density of amount of $1X10^6$ cells/ml
- Take 100K cells/well and plate the cells into Retronectin coated 24 well plate.
  (Note: after taking 100K cells/well, make up the volume of the $CD34^{+ve}$ cell culture medium up to 800ul in which 100K cells are plated).
- Transfer the cells to BSL2+ cell culture incubator and incubate cells for 30 min so that the cells settle down and attach to the Retronectin coating.
- ****Next steps should/must follow BSL2+ SOPs/Protocols*****
- During this incubation, bring ice in a container into BSL2+ BSC by surface sterilizing the container as per the BSL2+ SOPs. Also, take Polybrene stock solution into the BSL2+ BSC.
- Take the Lenti-OCT4 virus-containing vial from the BSL2+ freezer (-80°C) and bring the vial into the BSL2+ BSC upon surface sterilization as per the BSL2+ SOPs.
- Thaw the Lenti-virus encoding for OCT4 on ice in the BSL2+ BSC. Take the 24 well-plate from the incubator (after 30 min incubation) and transfer it to the BSL2+ BSC upon surface sterilization as per BSL2+ SOPs.
- Add 0.8ul of polybrene from the stock solution (10 mg/ml) to make 8 ug/ml final concentration (polybrene) to the cell culture medium.
- Carefully take the required (see next step) amount of Lenti-OCT4 viral solution (current batch of virus- 45ul) and add it on to the cells in 24 well plate by drop wise by using relevant pipette (P200 or P1000-depending on the volume needed to be taken) to make up 20 MOI.
- **The amount of Lenti-OCT4 solution to be taken depends on the viral titer. Depending on the batch of the virus and its titer the required amount of the virus to make up to 20 MOI varies.**
  (Note: after adding polybrene and virus particles, make up the volume up to 1ml with CD34+ cell culture medium.
- Mix the solution gently by using P1000 pipette.
- Take the 24 well cell culture plate out of the BSL2+ BSC and wrap the plate with saran wrap.
- Centrifuge the 24 well-plate at 1300 rpm for 90 min (using BSL2+ facility centrifuge) at RT.
- After the centrifugation, carefully remove the plate from the centrifuge and remove the saran wrap.
- Transfer the cells to the BSL2+ 37°C incubator and culture cells for 48 hrs.
- After 48 hour-post infection, Warm the CD34+ve cell culture medium by incubating it in the 37°C water-bath and then transfer the medium into BSL2+ BSC upon surface sterilization as per BSL2+ SOPs.
- Carefully take the 24 well plate out from the BSL2+ incubator and transfer the plate into the BSL2+ BSC post surface sterilization.
- Without disturbing the cells at the bottom of the plate, carefully collect the cell culture medium into a 15 ml falcon tube (Collect more than 90% of the medium (900ul of the medium).
  (Note: Use separate tubes for each well).
- Add 0.8 ml of fresh CD34+ve cell culture medium to the well.
- Close the 15 ml falcon tube by screw cap and centrifuge the tube at 1200 rpm for 10 min to pellet-down the cells.
- After centrifugation, surfaces sterilize the tube and transfer it into BSL2+ BSC.
- Carefully remove the cell culture medium leaving the cell pellet. Sometime, there may not a visible pellet.
- Add 700 ul of CD34+ve cell culture medium to the 15 ml falcon tube and resuspend the pellet and collect the solution by using P1000 pipette.
- Transfer this solution to the 24 well-plate.
- Transfer the cells to BSL2 incubator and culture the cells for 48 hrs.
- Next day after transferring infected cells to the BSL2 incubator, coat 6 well plate with 0.1% Gelatin solution (2 ml/ well of 6 well plate) for 30 min at 37 °C incubator.
- Take a frozen vial of irradiated Mouse embryonic fibroblast cells (iMEFs) ($1X10^6$ cells/vial) from liquid nitrogen tank and thaw as per the cell thawing SOP.

- Plate 200K cells in a well of 6 well-plate. Culture these iMEFs in DMEM medium containing 5% FBS for 24 hrs.
- Next day (48 hrs of post transferring infected cells in BSL2 incubator); warm the reprogramming medium in 37 °C water-bath. Upon surface sterilization and bring the medium into the BSC. Also take stock vial of BFGF into the BSC upon surface sterilization.
- Add 1ul/ml of BFGF (Stock - 10 ng/ul) to the required amount of reprogramming medium and mix the solution. (Note: for one well you would need 4ml of reprograming medium)
- Take-out the 6 well plate containing iMEFs and upon surface sterilization take it into the BSC.
- Aspirate the MEF culture medium with a Pasteur pipet that is connected to a vacuum suction.
- Add 1 ml of reprogramming medium to the iMEFs and rinse (remove the medium by Pasteur pipette/vacuum suction) the cells gently so that any remaining's of the MEF medium are removed.
- Add 1 ml of fresh reprogramming medium to iMEFs and transfer them back to BSL2 incubator.
- Take the 24 well plate with Lenti-OCT4 infected $CD34^{+ve}$ cells into the BSC upon surface sterilization. Also take cell dissociation buffer and PBS (warmed to 37°C) into the BSC after surface sterilization.
- Collect the cell culture medium and cells into a fresh/surface sterilized 15 ml falcon tube. (Note: Use separate tubes for each well).
- Add 500 ul of cell dissociation buffer to the well of 24 well-plate from which cell suspension was collected.
- Incubate the plate in the 37°C BSL2 incubator for 5 min. Take the plate out from the incubator, surface sterilize it and take it into the BSC. Add 1 ml of PBS to the well. Flush the cells. Collect the PBS + cell dissociation buffer + cells and add them to the 15 ml falcon tube containing remaining cells.
- Wash the well with 1 ml of PBS and collect the PBS and add it to the falcon tube.
- Spin down the cells at 1200 rmp for 10 min.
- Just before the spin is about to finish, take the iMEFs (6 well plate), surface sterilize it and take it into the BSC.
- Take the tube into BSC under sterile conditions and aspirate the solution without disturbing the pellet.
- Resuspend the cells in 2 ml of reprogramming medium and plate them onto iMEFs. The total volume of the medium in the 6 well plate will be 3 ml. Transfer the plate to the 37°C incubator (BSL2).
- Change medium with fresh reprogramming medium after 48 hrs. Check the cells prior to the cell culture medium change using bright-field microscope to visualize that the most of the cells are attached; (there may be few cells that are not attached).
- Pre-warm the reprogramming medium in falcon tube to 37°C and surface sterilize it and take it into the BSC. Also take BFGF stock containing tube into BSC upon surface sterilization.
- Add 1ul/ml of BFGF to the reprogramming medium and mix the solution. Take-out the 6 well plate containing $CD34^{+ve}$ cells infected with Lenti-OCT4 and surface sterilize it and take it into the BSC.
- Collect the medium into 15 ml falcon tube. Quickly add 1 ml of the reprogramming medium to the well and transfer the plate to the incubator. Spin down the cells (in the falcon tube-if there are any) by centrifuging the tube at 1200 rpm for 10 min.
- After the spin surface sterilize the tube and take it into the BSC. Bring the 6 well plate into the BSC upon surface sterilization.
- Aspirate the medium from the falcon tube using Pasteur pipette and resuspend the cells in 500 ul of reprogramming medium and add the volume to the 6 well plate (the well containing rest of the cells-iMEFs+OCT4-$CD34^{+ve}$ cells). Take 1.5 ml of reprogramming medium and rinse the 15 ml falcon tube and add the volume to the well containing rest of the cells-iMEFs+OCT4-$CD34^{+ve}$ cells making final volume of the medium to 3 ml (in each well).
- Take the plate back to the 37°C incubator (BSL2) and maintain the cells for 48 hrs.
- Upon 48 hr, prepare neural induction medium (3 ml-fresh/well). Pre-warm DMEM/F12 medium to 37°C in water-bath. Surface sterilize each reagent (bottle/vial/tube) and take each of them into the BSC.
- Prepare the neural induction medium by adding each component into the falcon tube.

- Take out the 6 well plate containing iMEFs+OCT4-CD34^{+ve} cells, surface sterilize the plate and take it into Pasteur pipette the BSC. Collect the cell culture medium from the 6 well plate by using pipette into a 15 ml falcon tube.
  (Note: Use separate tubes for each well).
- Gently, add 2 ml of neural induction medium to the well. Transfer the plate to incubator.
- Spin down the cells (if there are any) in the 15 ml falcon tube at 1200 rpm for 10 min.
- After the spin, surface sterilize the tubes and bring them into the BSC. Aspirate the medium from the 15 ml falcon tubes using glass Pasteur pipette. Use separate Pasteur Pipettes for each falcon tube. Re-suspend cells in 1 ml neural induction medium.
- Take the 6 well plate from the incubator and bring it into the BSC upon surface sterilization. Transfer the cells from each falcon tube to respective wells of 6 well plate.
- Transfer the 6 well plate back to the 37°C incubator.
- Replace the culture medium with a fresh neural induction medium on every 3rd day until the appearance of neural colonies.
- Between 2-3 weeks of addition of neural induction medium, neural colonies begin to appear and under bright-field microscope their morphology appears as follows in Fig.1C.
- The frequency of colonies that appear ranges from 0-4 colonies (100K cells CD34^{+Ve} cells infected with Lenti-OCT4)

Protocol for Neural colony picking and expansion

Reagents and initial setup:

- Poly ornithine solution (stock 0.01% solution) stored at 4°C
- Laminin solution (Stock 1mg/ml) stored at -20°C
- Neural precursor cell (NPC) medium:
  - DMEM/F12, Invitrogen
  - 1XN2, Invitrogen
  - 1XB27,Invitrogen
  - bFGF (20 ng/ml), R&D System
  - EGF (20 ng/ml), R&D System
  - LDN-193189 (100 nM), Stemgent
  - SB431542 (10 µM), Stemgent
  - CHIR99021 (3 µM) Stemgent
- Tissue culture (TC) treated 12 well plate
- Bright field Microscope (should be placed in biosafety cabinet)

Procedure:

- Between 2-3 weeks of addition of neural induction medium, neural colonies begin to appear and under bright-field microscope their morphology appears as shown in Fig.1C.
- The frequency of colonies that appear ranges from 0-4 colonies (100K cells $CD34^{+Ve}$ cells infected with Lenti-OCT4).
- Prior to picking neural colonies, coat required number of wells of 12 well plate with Poly-ornithine and Laminin.
  (Note: Number of wells would depend on number of colonies to be picked)
  - Protocol for coating plate with Poly-Ornithine and Laminin.
    - Take 12 well plate (TC treated), 1X PBS and Poly-ornithine solution (stored at 4° C) and surface sterilize them with 70% alcohol solution or as per BSL2 SOPs.
    - Bring them into BSC.
    - Dilute Poly-Ornithine solution 1:10 by using 1XPBS.
    - Add 500 µl of diluted Poly-Ornithine solution to each well of 12 well plate.
    - Transfer the pate to 37° C incubator and leave it in there for 2 hrs (Poly-Ornithine coating for 2 hrs).
    - Meanwhile, thaw Laminin solution (Stock: 1 mg/ml stored in -20° C) at 4° C.
    - Upon completion of Poly-Ornithine coating, take the plate from the incubator and after surface sterilization it and bring into BSC.
    - Surface sterilize the vial containing Laminin stock solution and bring it into the BSC.
    - Carefully remove the Poly-Ornithine solution from each well of 12 well plate.
    - Wash the wells with 1X PBS by gently releasing PBS solution (1000 µl) along with walls and remove the solution by vacuum suction or pipette.
    - Make 10 µg/ml Laminin working solution, by using 1X PBS.
    - Add 500 µl of 10 µg/ml Laminin solution to each well of 12 well plate. Coat required wells. Coat the wells that were previously coated with Poly-Ornithine.

- Take the plate out of BSC. Wrap the plate with Saran wrap.
- Transfer the plate to 4° C and coat the wells with Laminin at 4° C overnight.
- Next morning, unwrap the Saran wrap and surface sterilize the TC plate.
- Aspirate the Laminin solution and add 1 ml 1X PBS to the wells and the plate is ready to use.
- At this point the plate can be stored at 4° C for 2-3 days.

- Before colony picking, take images of each colony from each well in 6 well plate for reference.
- Colony picking requires the bright filed microscope to be taken into the BSC after surface sterilization with 70% Ethanol.
- Prepare NPC medium under sterile conditions.
- Have the 12 well plate with required number of wells coated with Poly-Ornithine/Laminin; ready.
- Remove the PBS from each well and Add 500 µl of NPC to each well of 12 well plate (that is coated with Poly-Ornithine/Laminin).
- Takeout the 6 well plate in which Lenti-OCT4 infected CD34+ve cells were plated in irradiated MEFs cultured for 2-3 weeks for the appearance of neural colonies, surface sterilize the plate and transfer it into BSC.
- Turn-on the bright field microscope. Take the 6 well plate onto the microscope and focus the microscope so that neural colonies are clearly visible in the field of view.
- Take a sterile p200 µl pipette tip and detach the colony from the edges by scraping at the colony edges.
- Once the colony almost detached from the substratum. Take a p1000 Pipette and aspirate the colony with minimal medium.
- Quickly is release/deposit the colony into a well of the 12 well plate that is coated with Poly-Ornithine/Laminin.
- Each colony should be detached from the master plate (6 well plate in which OCT-4 infected CD34+ve cells were plated on iMEFs) and plated into separate wells of 12 well plate (coated with Poly-Ornithine/Laminin).
- After transferring all the colonies into fresh 12 well plate. Transfer the plate into the 37° C incubator.
- Change the medium with fresh NPC medium every 3rd day.
- Once the cells are confluent, pass the cells into fresh plate that is coated with Poly-Ornithine/Laminin by passaging 1to 3.
- Assess the cells for NPC markers by FLOW cytometry and Immunostaining for NPC markers (SOX2, PAX6 and Nestin) and taking phase images for analyzing cell morphology.
- Once confirmed with the expression of the NPC markers, these colony derived cells could be expanded and differentiated into neurons of CNS and PNS.

Cryopreservation of iNPC-derived iNPC

Reagents and initial setup:

- DMEM/F12
- DMSO
- Accutase
- TrypLE
- NPC medium (DMEM/F12+1xN2, 1xB27, bFGF (20 ng/ml), EGF (20 ng/ml)
- Cryovials

Procedure:

- Perform all the experiments and procedure under BSL2 sterilization procedure and also follow BSL2 SOPs.
- Warm the DMEM/F12, Accutase, TrypLE and NPC media to 37° C in the water bath.
- Take the cell culture plate in which the iNPC are maintained out of the incubator.
- Surface sterilize the plate and take it into the BSL2 BSC.
- Add 300 µl /1 ml of TrypLE to each well of 24 well plate / each well of 6 w plate respectively.
- Take a 15 ml falcon tube and surface sterilize and take it into the BSL2 BSC and label it as singularized iNPC.
- Transfer the plate back to the incubator and leave it in there for 5 min.
- After 5 min take the plate in to the BSL2 BSC and collect the TypLE by using p1000 and dispense it into the 15 ml falcon tube.
- Add 3 ml of DMEM/F12 medium to the 15 ml falcon tube.
- Next add 500 µl /1.5 ml of Accutase to each well of 24 w plate / each well of 6 w plate respectively.
- Transfer the plate into the incubator for 5 min.
- After 5 min visualize the cells under the bright field microscope. By now the cells should/about to detach and appear round.
- Take the plate into the BSL2 BSC and gently remove the Accutase solution and dispense it into the 15 ml falcon tube containing TrypLE and DMEM/F12 medium.
- Take 1 ml /3 ml of fresh DMEM/F12 medium for each well of 24 w or 6 w plate and dispense the volume into each well respectively.
- Gently flush the cells and collect them into the 15 ml falcon tube.
- Once all the cells are collected, wash the well with additional 500 µl / 1ml of DMEM/F12 per each well of 24 w /6 w plate respectively and combine the volumes in the 15 ml flacon tube.
- Screw the cap of the 15 ml falcon tube. Centrifuge the cells at 1500 rpm for 5 min at RT.
- After the centrifugation, surface sterilize the falcon tube and take it into the BSL2 BSC.
- Aspirate the supernatant without disturbing the cell pellet.
- Resuspend the cells in 1 ml (if the cells were taken from couple of wells of 24 well plate) or 5 ml of the sensory neuron maintenance medium if the cells are taken from 2-3 wells of 6 w plate.
- Resuspend gently and make sure that there are no clumps of cells.
- Take 50 µl of cell suspension and count the cells using cell countess or hemocytometer.
- Take desired number of cryovials surface sterilize them and take them into BSL2 BSC and label the vials.
- Add DMSO to the cell suspension so that the final concentration of DMSO is 10%.
- Compensate the cell number for the increased cell volume due to the addition of DMSO.

- Calculate the number of cells in each ml of the cell suspension.
- Add 1.5 ml of cell suspension and label each vial with the number of cells in each vial.
- Screw caps for each vial and transfer the vials into Mr. Frosty (cell freezing box/container).
- Transfer the Mr. Frosty (cell freezing box/container) to -80° C.
- Leave the Mr. Frosty (cell freezing box/container) to -80° C overnight.
- Next day, move the cells to liquid nitrogen storage.

Differentiation of iNPCs (OCT4 –derived NPCs) to Sensory neurons

Reagents and initial setup:

- Matrigel (already diluted 1:1 with KO-DMEM and stored at -30° C)
- Accutase and all media below should be pre-warmed at 37° C.
- NPC medium (DMEM/F12+1xN2, 1xB27, bFGF (20ng/ml), EGF (20ng/ml)
- Sensory neuron specification medium
    - DMEM/F12+ 1xN2+ 1xB27, Invitrogen
    - N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester DAPT (10 µM) Sigma
    - SU5402 (1 µM)
    - CHIR99021 (3 µM) Stemgent
- Sensory neuron maturation medium
    - DMEM/F12+ 1xN2+ 1xB27
    - NGF (10 ng/ml)+ BDNF (10 ng/ml) + NT3 (10 ng/ml)+ GDNF (10 ng/ml) R&D systems
    - Ascorbic acid (200 µM)
    - Forskolin (5 µM)
- 24 well plate (Tissue culture treated)

Procedure:

- For differentiating iNPCs to Sensory neurons, coat 24 well plate with Matrigel (1 ml of Matrigel diluted in Knockout DMEM to make 15 ml of working stock Matrigel solution).
    - Perform all the experiments under BSL 2 surface sterilization conditions and by following BSL 2 SOPs.
    - Use TC treated 24 well plate
    - Dilute the Matrigel (take the stock solution of Matrigel from the -30° C and let it thaw at RT/4° C) in Knockout DMEM 1:15 ratio (by adding 14 ml of KO DMEM to 1 ml of Matrigel stock solution).
    - Add 300 µl of working stock solution of Matrigel to each required well of 24 well plate.
    - Coat the plate with matrigel by incubating at 4° C for overnight or 2-3 hr at RT.
      (Note: prolong coating of Matrigel at RT is not recommended)
- Prepare NPC medium under sterile conditions and warm the medium to 37° C in the water bath.
- Warm Accutase solution and sterile 1X PBS solution to 37° C in the water bath.
- Take the NPC medium, Accutase solution and sterile 1X PBS solution into BSC upon surface sterilization.
- Take the 12 well plate in which iNPCs are being cultured into BSC upon surface sterilization and remove the culture medium by vacuum suction.
- Add 500 µl of Accutase solution to each well of iNPC containing well.
- Transfer the plate to the incubator for 4-5 min.
- Check the cells under the bright field microscope. If the cells appear firmly attached to the well, then transfer cells to the 37°C incubator for additional 2-3 min incubation.
- After the time point, observe the cells under the bright field microscope again.
- If the cells appear rounded up and about to detach from the substratum, surface sterilize and bring the plate into the BSC.
- Take a fresh 15 ml falcon tube and surface sterilize it and take it into the BSC.
- Using a P1000 pipette collect the Accutase solution into the 15 ml falcon tube.

- Then add 1 ml of 1X PBS to the well by using 5 ml pipette.
- Using P1000 Pipette, flush the cells very gently and collect cells into the falcon tube to combine Accutase solution and 1X PBS solution containing iNPCs.
- Centrifuge the cells at 1500 rpm for 5 min to pellet them.
- After centrifugation, bring the cells that are in the 15 ml falcon tube into the BSC upon surface sterilization.
- Aspirate the supernatant (PBS+accutase solution) by vacuum suction.
- Resuspend the cells in 5 ml of NPC medium.
- Count the cells (cell countess/hemocytometer) by taking 20 -50 µl of cell suspension.
- Take the 24 well plate into which iNPCs will be plated, remove the matrigel solution and add 300 µl of NPC medium.
- Plate 100K cells into each well of 24 well plate.
- Make sure that the cells are evenly distributed in the wells.
- Transfer the plate to the 37°C incubator.
- Check the cells under bright field microscope next day, and the cells should cover 90% of the well. If not wait for one more day.
- Prepare sensory neuron specification medium under sterile conditions and warm the medium to 37°C by placing into the water bath.
- Take the 24 well plate containing iNPCs and the sensory neuron specification medium into the BSC upon surface sterilization.
- Remove the NPC medium from each well and replace the medium with 300 µl of sensory neuron specification medium.
- Replace the medium with the fresh sensory neuron specification medium every 2nd day for a week.
- This should lead the differentiation of iNPCs to sensory neurons.
- After a week of differentiation, replace the medium with sensory neuron maturation medium and change medium here after every 2nd- 3rd day.

Maintenance of iNPCs derived iSN (sensory neurons)

Reagents and initial setup:

- Sensory neuron maturation medium
  - DMEM/F12+ 1xN2+ 1xB27
  - NGF (10 ng/ml)+ BDNF (10 ng/ml) + NT3 (10 ng/ml)+ GDNF (10 ng/ml) R&D systems
  - Ascorbic acid (200 µM)
  - Forskolin (5 µM)

Procedure:

- After the completion of treatment with small molecules to derived sensory neurons (iSN), replace the culture medium with sensory neuron maintenance medium.
- From this point after the medium changes will be done on every 3rd day.
- Warm the DMEM/F12 cell culture medium to 37° C in the water bath.
- On the 3rd day, under the BSL2 sterile conditions, prepare 2X sensory neuron maintenance medium containing double the amount of all the supplements and neural factors (2x).
- Take out the tissue culture plate (24 well plate) containing differentiated sensory neurons and bring it into the BSL2 hood upon surface sterilization.
- Carefully remove 50% of the culture medium from each well by using p1000 pipette.
- Add equal amount of the 2x sensory neuron maintenance medium to each well carefully.
- Perform these medium changes on every 3rd day until the neurons reach 21 days post differentiation.
- These neurons are ready for analysis for functional assays or analysis for sensory neuron functional markers.

Seeding iSN-sensory neurons into 96 well format

Reagents and initial setup:

- Matrigel (already diluted 1:1 with KO-DMEM and stored at -30° C)
- Accutase and all media below should be pre-warmed at 37° C.
- 96 well plate (TC-treated/TC-grade)
- TrypLE
- Y-27632 (ROCK inhibitor, ROCKi) (10 µM)
- Sensory neuron maturation medium
    - DMEM/F12+ 1xN2+ 1xB27
    - NGF (10 ng/ml)+ BDNF (10 ng/ml) + NT3 (10 ng/ml)+ GDNF (10 ng/ml) R&D systems
    - Ascorbic acid (200 µM)
    - Forskolin (5 µM)
- Differentiated and matured iSNs

Procedure:

- All the Experiments and methods should be performed under BSL2 SOPs and sterilization conditions.
- Two days prior to the plating iSN into 96 well plates, take a 96 well plate and take it into the BSL2, BSC upon surface sterilization.
- Takeout the Matrigel stock solution from the -20 freezer. Thaw the Matrigel at 4° C. Take KO-DMEM from the 4° C fridge and bring it into the BSL@ BSC upon surface sterilization.
- Dilute the Matrigel 1:15 with KO-DMEM.
- Add 100-150 µl of Matrigel solution to the each well (required number of wells).
- Take the plate out of the BSL2 BSC and wrap the plate with Saran Wrap.
- Let the Matrigel settle down/let the matrix to form by incubating the plate over night at 4° C fridge.
- Next day morning, remove the Saran wrap from around the plate and surface sterilize the plate and take it into the BSL2 BSC.
- With the vacuum suction Pasteur pipette aspirate the Matrigel solution. This has to be done carefully so that only the solution should be aspirated.
- Once Matrigel from all the wells (required) aspirated, let the Matrigel dry by leaving the plate in the hood (remove the lid of the plate and put it aside).
- Dry the plate (s) for 4-5 hr so that the Matrigel in the wells is completely dry (confirmed by visual observation).
- After drying, coat the same wells with Matrigel for an additional Matrigel coating by following the steps 3 to 7.
- The next day the plate is ready for plating iSN.
- Prepare the sensory neuron maintenance medium.
- Aspirate the Matrigel solution carefully and add 50 µl of sensory neuron maturation medium to each well.
- Takeout the plate that containing iSN – 24 w or 6w plate from the BSL2 incubator.
- Take it into the BSL2 BSC upon surface sterilization.
- Aspirate the culture medium from each well and replace with 300 µl (24w) or 1 ml (6w) of TrypLE to each well.
- Take a 15 ml falcon tube and surface sterilize and take it into the BSL2 BSC and label it as singularized iSN.

- Transfer the plate back to the incubator and leave it in there for 5 min.
- After 5 min take the plate in to the BSL2 BSC and collect the TypLE by using p1000 and dispense it into the 15 ml falcon tube.
- Add 3 ml of DMEM/F12 medium to the 15 ml falcon tube.
- Next add 500 µl /1.5 ml of Accutase to each well of 24 w plate / each well of 6 w plate respectively.
- Transfer the plate into the incubator for 5 min.
- After 5 min visualize the cells under the bright field microscope. By now the cells should/about to detach and appear round.
- Take the plate into the BSL2 BSC and gently remove the Accutase solution and dispense it into the 15 ml falcon tube containing TrypLE and DMEM/F12 medium.
- Take 1 ml /3 ml of fresh DMEM/F12 medium for each well of 24 w or 6 w plate and dispense the volume into each well respectively.
- Gently flush the cells and collect them into the 15 ml falcon tube.
- Once all the cells are collected, wash the well with additional 500 µl / 1ml of DMEM/F12 per each well of 24 w /6 w plate respectively and combine the volumes in the 15 ml flacon tube.
- Screw the cap of the 15 ml falcon tube. Centrifuge the cells at 1500 rpm for 5 min at RT.
- After the centrifugation, surface sterilize the falcon tube and take it into the BSL2 BSC.
- Aspirate the supernatant without disturbing the cell pellet.
- Resuspend the cells in sensory neuron maturation medium plus 10µM ROCKi. Use 1 ml (if the cells were taken from two wells of 24 well plate or 5 ml of if the cells are taken from 2-3 wells of 6 w plate.
- Resuspend gently and make sure that there are no clumps of cells.
- Take 50 µl of cell suspension and count the cells using cell countess or hemocytometer.
- Adjust the cell suspension using sensory neuron media plus 10µM ROCKi to achieve a cell density of 10Kcells per 100µl.
- Add cell suspension to a reagent trough and use a multi-channel pipette to seed the matrigel pre-coated 96 well plate and put in incubator.
- Replace media after 24hr with SN-media minus ROCKi. From this point, maintain the cells as per the Maintenance of iSN protocol for 14 days.