iMPS PLATEDOWN PROCEDURE

1. PURPOSE
   Plate down iMPS in order to differentiate into iMNs.

2. RESPONSIBILITIES
   All staff working with the iMPS that have been sent by the Cedars-Sinai Motor Neuron Center in the Svendsen lab.

3. PROCEDURE

3.1 *Plate and media preparation*

Materials

3.1.1 Laminin (CAS 114956-81-9, Sigma, L2020-1ML), 1 mg/mL

3.1.2 1XPBS, -/- (Various distributers)

3.1.3 Tissue culture-treated plate(s) (use appropriate plate for assay)

3.1.4 50 mL conical(s)

3.1.5 Serological pipettes

3.1.6 Multi-channel micropipettor (for 96 well plates) with tips and reservoirs

3.1.7 STAGE 1 Media

Plate Coating

3.1.8 Prepare 1X laminin (50 µg/mL) by combining 1 mL of stock laminin (1 mg/mL) with 19 mL sterile 1XPBS in a 50mL conical.

3.1.9 Aliquot 1 mL/aliquot and store at -20°C for up to one year. 1X laminin aliquots are good for up to two weeks at 4°C after thaw.

3.1.10 Coat plate with laminin. Leave in incubator 37°C for 1 - 2 hours.

Table 1 – Laminin coating volume

Plate Type	**1X Laminin/well**
6-well	1 mL
24-well	500 µL
96-well	50 µL

3.1.11 During the laminin incubation, prepare STAGE 1 Media, see media formulation (Appendix A). Store at 4°C for up to one month.

3.2 *Cell Platedown procedure*

Materials

3.2.1 Waterbath, 37°C
3.2.2 Centrifuge, swing-bucket
3.2.3 Tissue culture-treated plate(s) (use appropriate plate for assay)
3.2.4 STAGE 1 Media (Appendix A)
3.2.5 Dry ice
3.2.6 Cryopreserved cell vials
3.2.7 15 mL conical(s)
3.2.8 Serological pipettes
3.2.9 Multi-channel micropipettor (for 96 well plates) with tips and reservoirs

Protocol

3.2.10 Decontaminate the hood and materials.
3.2.11 In a water bath, thaw cell vial **one at a time**. Swirl the vial in a figure-8 formation until only a small ice crystal remaining (~0.5 cm in diameter). **Do not let the vials thaw completely**.
3.2.12 Aseptically transfer the vial to the BSC and use a 1, 2 or 5 mL serological pipette to transfer the cells into a 15 mL conical. In a drop-wise fashion, add 9 mL of STAGE1 media . (Do this slowly so to prevent cell shock).
3.2.13 Centrifuge cells at 300 x g (rcf) at 4°C for 5 minutes.

3.2.14 Aspirate media and discard, then re-suspend pellet in approximately 2 mL of STAGE 1 media.

3.2.15 Count cells using the method listed in step 3.2.16. It is not recommended to count iMPS with an automated system as these cells exhibit a wide range of sizes and shapes, which can create inaccuracies when using the automated counters.

NOTE: If seeding multiple cell lines at one time, leave conical on ice until all remaining cell lines are counted and the correct dilutions are calculated.

3.2.16 Count the viable cells with an inverted bright field microscope at 10X using phase contrast. Count the live/dead cells in the four large corner squares in both chambers as demonstrated in Figure 1 and Figure 2. If there is not at least 75 total cells counted in the four corner squares, re-dilute the cell suspension and count again. Live/viable cells are trypan blue negative and dead cells are trypan blue positive.

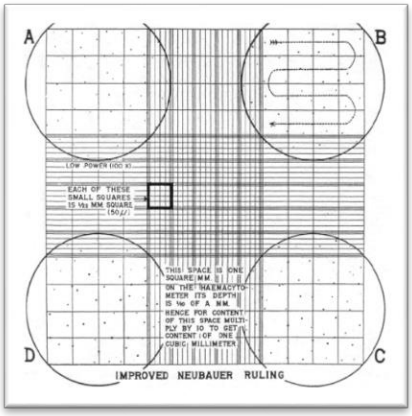

Figure 1: Hemacytometer Fields

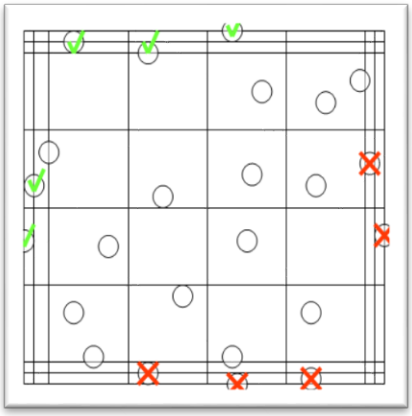

Figure 2: Cell Counting Criteria

3.2.17 Calculate the % viability and viable cell concentration. Use equation below for viable cell concentration.

$$\frac{viable\ cells\ counted}{total\ squares\ counted} \times 10{,}000 \times dilution\ factor = {}^{viable\ cells}/_{mL}$$

3.2.18 Calculate the dilution factor required for seeding listed as listed in Table 2. Dilute the cell concentration appropriately using STAGE 1 media.

Table 2 – Seeding concentration and volume

Plate Type	Seeding cell concentration	Volume/well
6-well	360,000 cells/mL	2 mL
96-well	400,000 cells/mL	0.1 mL

3.2.19 Seed wells, using the appropriate volume listed in Table 2. It's best to use a multichannel micropipette when seeding a 96 well plate. Mix gently throughout the seeding process to ensure consistent cell concentration across all wells.

3.2.20 Differentiate the cells at 37°C, 5% CO_2 in a humidified incubator. Based on current methods the differentiation time is 21 days. However more cell aggregation or 'clumping' has been observed when differentiating past 14 days. If single cell analysis is required, it may be appropriate to run a 14 day differentiation.

3.3 *iMN Differentiation*

Materials

3.3.1 STAGE 1 Media

3.3.2 STAGE 2 Media

3.3.3 Serological pipettes

3.3.4 Multi-channel micropipettor (for 96 well plates) with tips and reservoirs

3.3.5 Paraformaldehyde (PFA)

3.3.6 1XPBS (-/-)

3.3.7 Prepare a 4% solution of PFA in 1XPBS

Procedure

Day 0 - 6

3.3.8 Perform a full media exchange every 2 – 3 days by removing the spent media and adding fresh STAGE 1 media (volume in Table 2). Remove/transfer media very carefully as to not lift the cells off of the plate. Feed cells a minimum of two times before day 7.

Day 7 +

3.3.9 Remove all STAGE 1 media from each well and transition the differentiation to STAGE 2 media (volume in Table 2). Exchange all media every 3-4 days with fresh STAGE 2 media.

Assay timing, fixation or cell pellet harvest

* Cell imaging has shown to be more reliable on day 14, however there are fewer mature motor neurons at this timepoint. If the assay required single cell resolution you may begin the live cell assay at this timepoint (discuss with supervisor) or fix cells for assay(s). For protein or nucleic acid studies, the cells have traditionally been differentiated to day 21 and harvested as cell pellets.

3.3.10 *Fixation* – Remove the media from each well. Rinse each well once with sterile 1XPBS. Transfer 4% PFA/1XPBS onto each well. Incubate for 15 - 25 minutes at room temperature. After incubation, discard the PFA and rinse each well once with 1XPBS. Transfer the appropriate volume of 1XPBS for storage. Reference Table 3 for volumes. Store fixed plates at 4°C.

3.3.11 *Cell Pellets* – Remove the media from each well. Rinse each well once with sterile 1XPBS and discard wash. Transfer 0.5 mL of 1XPBS into each well. Mechanically lift the cells from the dish using a cell scraper or other tool such as a sterile, inverted P1000 micropipettor tip. Transfer all cells to a conical tube, mix and aliquot into microcentrifuge tubes. Pellet by centrifugation for 5 minutes at 300 x g, 4°C. Snap freeze in LN_2, store at -80°C.

Table 3 – Fixation process volumes

Plate Type	Fixation Volume	Rinsing Volume	Storage Volume
6-well	2 mL	1 mL	3 mL
96-well	0.05 mL	0.05 mL	0.2 mL