THAWING DAY 12 diMNs CRYOVIAL

1. SHIPPING CONDITIONS

Day 12 diMNs are cryopreserved as a single-cell suspension in 1.5mL cryovials. They are frozen in 1mL aliquots using Cryostor CS10.

2. UPON DELIVERY

Immediately transfer your cryovials to an appropriate cryogenic storage system.

3. MATERIALS

Materials	Catalog Numbers	Company
IMDM	12440061	Life Technologies
F12	11765062	Life Technologies
B27 (with vitamin A)	17504044	Life Technologies
N2	17502048	Life Technologies
NEAA	11140050	Life Technologies
Antibiotic Antimycotic	15240062	Life Technologies
SAG (Sonic Hedgehog Agonist)	11914	Cayman Chemical
All-Trans Retinoic Acid (RA)	04-0021	Stemgent
BDNF	450-02	Peprotech
GDNF	450-10	Peprotech
Ascorbic acid	A4403	Sigma-Aldrich
Compound E	565790	Calbiochem
DAPT	13197	Cayman Chemical
db-Camp	28745	Sigma-Aldrich
Rock Inhibitor (Y-27632)	72308	StemCell Technologies
Growth Factor Reduced Matrigel	354230	Corning

4. MEDIA PREPARATION

NOTE: Protect Stage 3 media from light and store at 4°C.

Stage 3 Media (used for D12-D32)					
Component	Stock Concentration	Final Concentration	Commonly used volumes:		
			1L	500mL	250mL
IMDM	1X	47.5%	475mL	237.5mL	118.75mL
F12	1X	47.5%	475mL	237.5mL	118.75mL
NEAA	100X	1%	10mL	5mL	2.5mL
B27	50X	2%	20mL	10mL	5mL
N2	100X	1%	10mL	5mL	2.5mL
Anti/Anti	100X	1%	10mL	5mL	2.5mL
SAG	100mM	0.1µM	1µL	0.5µL	0.25µL
db-cAMP	10mM	0.1µM	10µL	5µL	2.5µL
All-trans RA	10mM	0.5µM	50µL	25µL	12.5µL
Compound E	1mM	0.1µM	100uL	50uL	25µL
DAPT	20mM	2.5µM	125µL	62.5µL	31.3µL
Ascorbic Acid	500µg/mL	200ng/mL	400µL	200µL	100µL
BDNF	10µg/mL	10ng/mL	1mL	500µL	250µL
GDNF	10µg/mL	10ng/mL	1mL	500µL	250µL
Include in Stage 3 media for *thaw only*:					
Rock I (Y-27632)	10mM	10µM	1mL	500µL	250µL

5. PROCEDURE

5.1. PREPARE BEFORE THAW

5.1.1. Matrigel coated tissue culture plates

NOTE: We recommend you coat your plates with a concentration of 1mg GFR-Matrigel in 6mls of basal media.

5.1.2. Stage 3 Media with Rock Inhibitor (ST3+Rocki). Use at room temperature. Do not warm media in water bath.

5.2. THAWING

5.2.1. Set out Matrigel plates for a minimum of 1 hour before thawing.

5.2.2. Remove cells from cryogenic storage and begin thaw immediately.

5.2.3. Warm D12 diMNs cryovial in 37°C water bath for ~2 minutes.

5.2.4. Remove vial from water bath when only a small ice crystal remains.

5.2.5. Thoroughly spray vial with 70% isopropyl alcohol or ethanol and transfer to a biosafety cabinet.

5.2.6. Using a sterile 2mL serological pipet, gently transfer the contents of the cryovial drop-wise (~1-2 drops/sec) to a sterile 15mL conical tube containing 8mL of ST3+Rocki.

5.2.7. Rinse the empty cryovial with 1mL of ST3+Rocki and add this drop-wise to the 15mL conical tube containing the D12 diMNs suspension.

5.2.8. Cap the 15mL conical and gently invert the tube 3-5 times.

5.2.9. Spin at 350g for 5min at room temperature.

5.2.10. Aspirate supernatant without disturbing the pellet.

5.2.11. Flick pellet.

5.2.12. Resuspend pellet in 1mL ST3+Rocki (gently triturate with a P1000 pipet to homogenize pellet).

5.2.13. Count cells (adjust the final volume using ST3+Rocki as appropriate for your cell counting system).

5.2.13.1. Determine seeding density appropriate for your application.

Note: We have successfully plated D12 diMNs at the following densities:

- $1.0x10^5$ cells/cm^2
- $5.0x10^4$ cells/cm^2
- $2.5x10^4$ cells/cm^2

5.2.14. Aspirate Matrigel from cell culture plate(s).

5.2.15. Plate cells at your desired target plating density. Use ST3+Rocki as the plating medium at volumes appropriate for your plating vessel.

5.2.16. 24 hours after plating, replace 100% of ST3+Rocki with fresh Stage 3, no Rock Inhibitor (ST3).

5.2.17. Change ST3 every 48 hrs.

5.2.17.1. When changing media, aspirate ~75% of old media

5.2.17.2. Feed fresh ST3 in a slow, drop-wise manner

IMPORTANT: As the cultures mature the neurons become increasingly fragile. Gentle drop-wise feeding helps keep neurons intact and attached to the well.

5.2.18. Continue to incubate cultures at 37°C, 5% CO_2 for the entirety of the differentiation process.

5.2.19. See below for example brightfield image of D12 diMNs 24hrs post thaw.

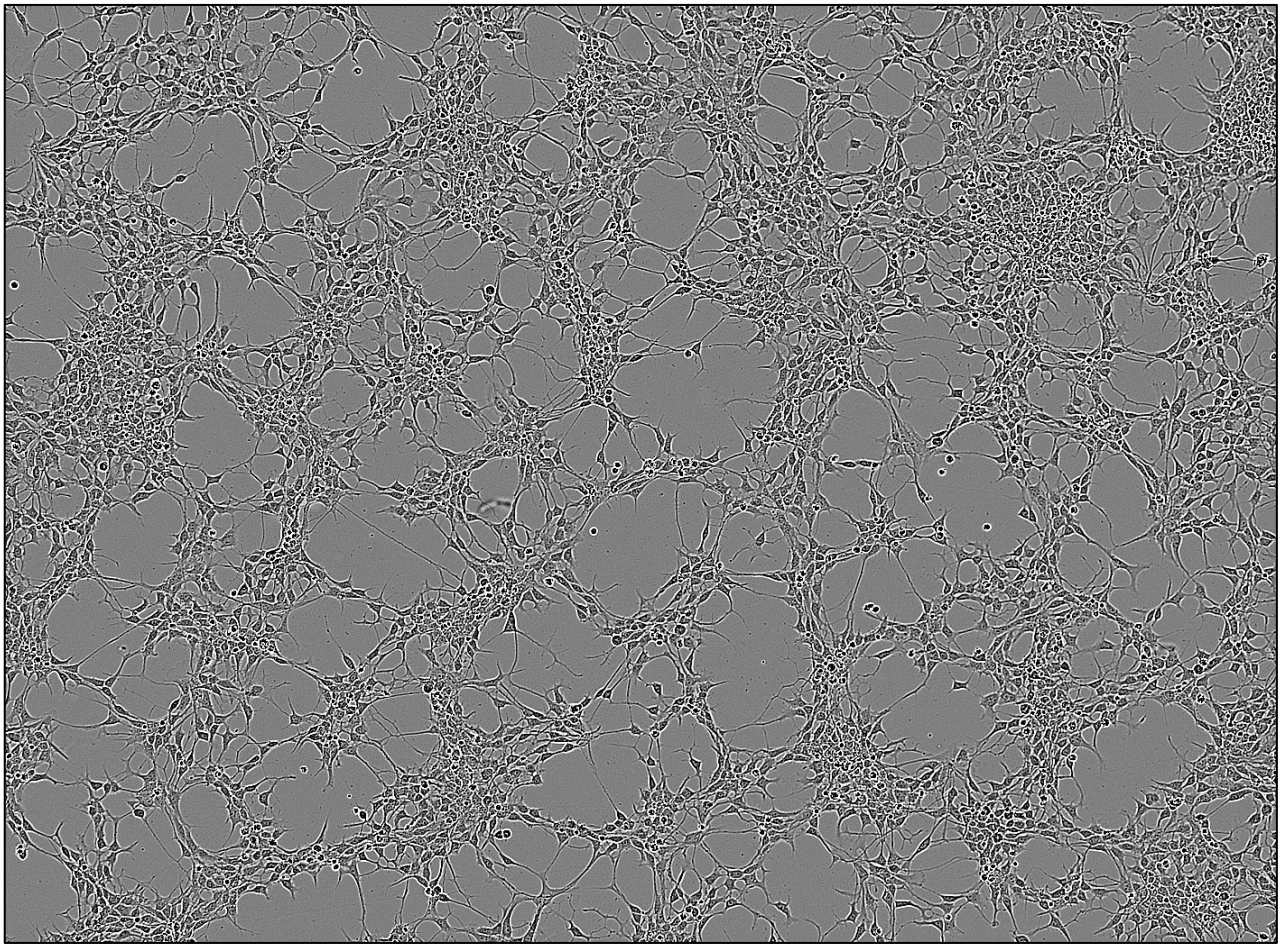

Figure 1. 10X Brightfield image of excellent attachment of D12 diMNs 24 hrs post thaw, following two PBS washes and media change.