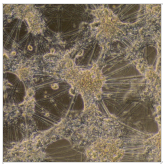

Jul 23, 2020

Dissociation of neuronal culture to single cells for scRNA-seq (10x Genomics)

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bh32j8qe

Cellular Generation and Phenotyping

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ABSTRACT

This protocol outlines a method for dissociating a human pluripotent stem cell-derived neuronal culture to single cells for loading onto a Chromium 10x chip for single cell RNA-sequencing.

This protocol makes a distinction between **early neuronal progenitors** and **mature** neuronal cultures, as additional steps and reagents are required in order to sufficiently dissociate the latter. These include:
- DNase Vial (D2)
- PDS Kit Papain Vial

Note: In our labs, iPS cells were undergoing a 52-day long differentiation process to dopaminergic neurons (adapted from doi.org/10.1038/nature10648), and were treated under the 'mature' conditions when harvested from day 20 onward, and as 'early neuronal progenitors' on day 11. Example images of neuronal culture from our labs can be found in the Guidelines of this protocol for reference.

This protocol assumes use of:
- 12-well tissue culture plates (3.8 cm^2 surface area per well) for samples/wells being harvested for dissociation (see Materials).
- A NucleoCounter®NC-200™ for purposes of cell counting.

DOI

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PROTOCOL CITATION

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KEYWORDS

neuronal, dissociation, 10x, single-cell, cell-culture, scRNA-seq, neurons

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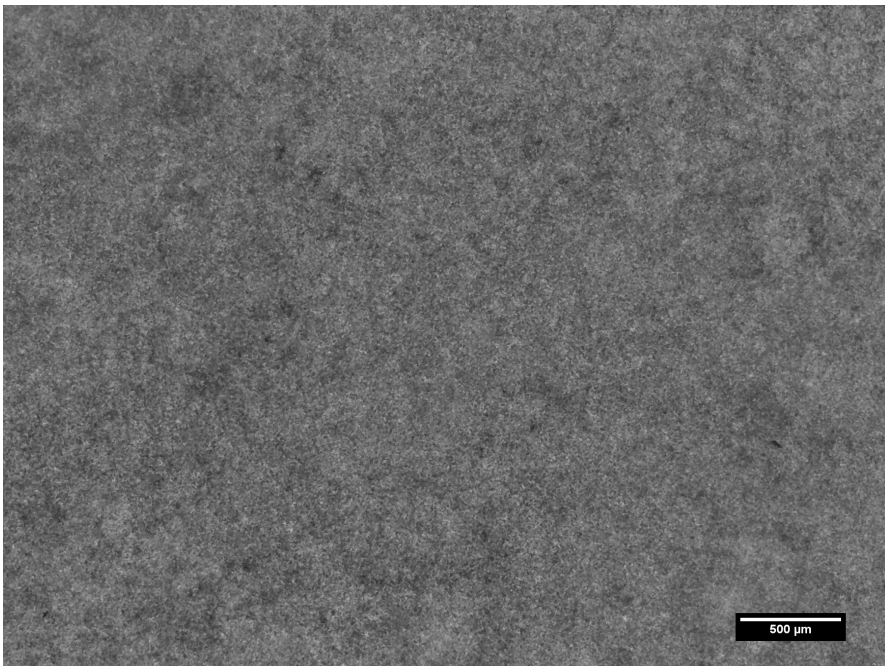
GUIDELINES

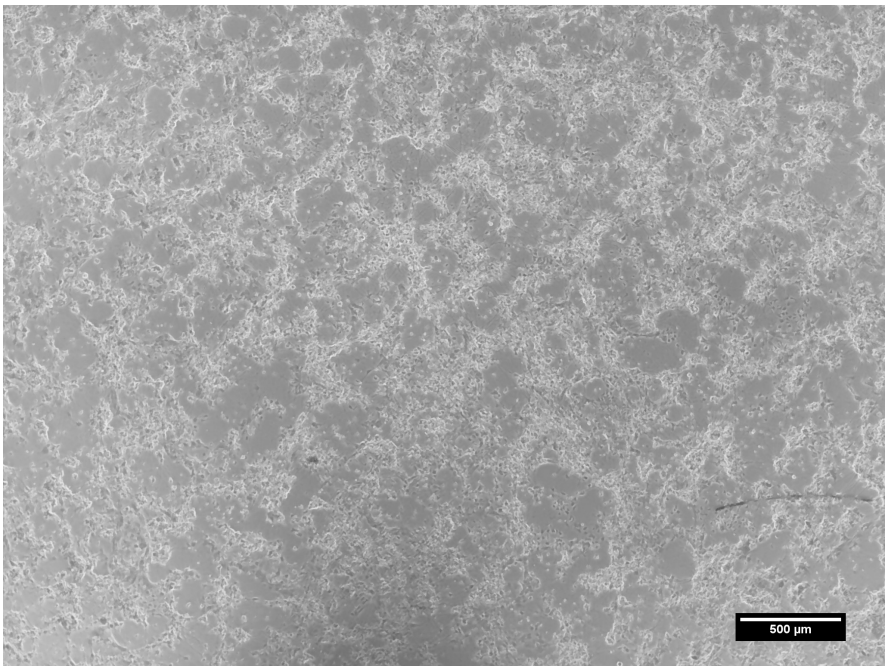
Unless otherwise stated, all steps should be performed under sterile conditions in a CL2 biological safety cabinet.

Refer to 10x Genomics Chromium single cell gene expression kit guidelines for subsequent steps in loading cells.

Below are example images showing a neuronal culture undergoing a 52-day differentiation to dopaminergic neurons. These are for reference regarding dissociation steps in this protocol that differ for early neuronal progenitors and mature neurons. Details on the protocol used can be found in the Abstract of this protocol.



Example of neuronal culture at **Day 11** of differentiation



Example of neuronal culture at **Day 30** of differentiation

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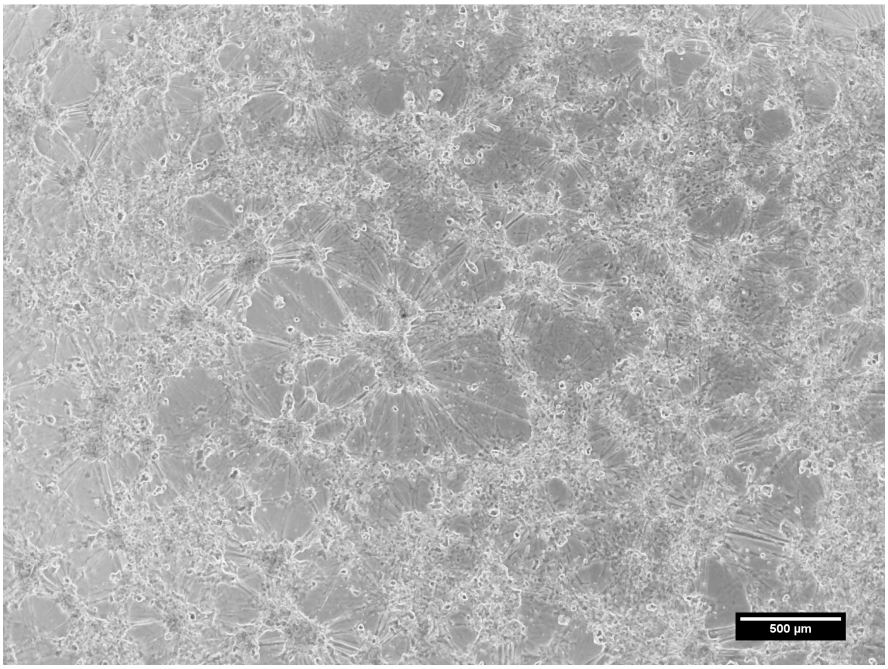

Example of neuronal culture at **Day 52** of differentiation

MATERIALS

NAME	CATALOG #	VENDOR
Ice		
DNA LoBind Tubes, 1.5 mL	0030108051	Eppendorf
DPBS	14190	Invitrogen - Thermo Fisher
Falcon™ 15mL Conical Centrifuge Tubes	14-959-53A	Fisher Scientific
DMEM/F-12, GlutaMAX™ supplement	10565018	Thermo Fisher
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher
Y-27632 dihydrochloride	Y0503	Sigma – Aldrich
DNase Vial (D2)	LK003170	Worthington Biochemical Corporation
PDS Kit Papain Vial	LK003176	Worthington Biochemical Corporation
PluriStrainer Mini 40µm	43-10040-60	pluriSelect
1.5 ml TubeOne® Microcentrifuge Tubes Natural (Sterile)	S1615-5510	StarLab
Bovine Serum Albumin	A0281	Sigma
Water for embryo transfer sterile filtered	W1503-500ML	Sigma Aldrich
0.6ml Crystal Clear Microcentrifuge Tubes (Sterile)	E1405-0610	StarLab
12-well Falcon™ Polystyrene Microplates	10489482	Fisher Scientific

MATERIALS TEXT

Equipment:

Centrifuge (for both 15mL & 1.5mL tubes)
Pipette boy
Sterile 5/10mL stripettes
P1000 pipette and filter tips

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Vacuum aspirator and tips
Microbiology Safety Cabinet (MSC)
Light Microscope
Scale (for making up BSA)
Method of Cell Counting (NucleoCounter®NC-200™)
37 °C , 5% CO_2 incubator

SAFETY WARNINGS

Rock inhibitor (Y-27632) - Harmful if swallowed, in contact with skin or inhaled.

BEFORE STARTING

Prepare a 10% BSA solution **100 mg/ml** by dissolving Bovine Serum Albumin powder in sterile Water for Embryo Transfer (or equivalent water for cell culture). Filter sterilise with a **0.2 µm** sterilising grade filter. Aliquot into sterile 0.6 mL microcentrifuge tubes. Store at **-20 °C** . Thaw at **Room temperature** and resuspend prior to use.

- Pre-warm Accutase to **37 °C** shortly before starting *(Caution: Accutase is inactivated after 45 minutes at* **37 °C** *)*
- If working with mature neurons, calculate how many vials of Papain/DNase you will need given the number of wells being harvested.

Buffer Preparation

1 Prepare **Wash Buffer 1 (without DNase)** by supplementing 15mL DMEM:F12 + GlutaMAX with ROCK inhibitor (Y-27632) to a final concentration of 10µM.

Note: We make up 15mL of ***Wash Buffer 1*** *for every 6 wells of a 12 well plate to dissociate.*

For mature neurons, 1 vial DNase (D2) should be added for every 15mL of buffer prepared. However, ***DNase should only added immediately prior to use of the buffer*** *when the cells are nearing the end of their dissociation. Keep the DNase at* **4 °C** *until use.*

Cells	DMEM/F12 + glutamax	Rock inhibitor (Y-27632) 10mM stock	DNase (D2)
Neuronal progenitors (~Day 11)	15 mL	15 uL	Not required
Mature Neurons (> Day 20)	15 mL	15 uL	1 vial (250uL)

Wash Buffer 1 composition for cells at different stages

2 Prepare **Wash Buffer 2** by adding 10% BSA solution (100 µg/mL) to DPBS (-/-) to a final concentration of 400 µg/mL. i.e. add 60 µL 10% BSA solution to 15 mL of DPBS (-/-).

3 Prepare **Dissociation Buffer for mature neurons** by combining Accutase with DPBS (-/-) at a 1:1 ratio. Add 1 vial Papain (PDS Kit) per 5mL buffer. Reconstitute the lyophilised papain with the Accutase/DPBS solution, replace the lid on the vial and invert several times ensuring all powder in the vial and on the lid is dissolved. Transfer solution back into the Dissociation Buffer tube.

Dissociation Buffer for neuronal progenitors is undiluted Accutase only.

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Cells	Accutase	DPBS (-/-)	Papain (PDS kit)
Neuronal progenitors (~Day 11)	0.5 mL per well	Not required	Not required
Mature Neurons (> Day 20)	2.5 mL	2.5 mL	1 vial

Dissociation Buffer composition for cells at different stages

Dissociation

4 Aspirate the media from the well(s) of the neuronal culture and gently add **1 mL** of DPBS (-/-) without disturbing the cell layer.

5 Aspirate the DPBS (-/-) and add **0.5 mL** of **Dissociation Buffer** to each well.

6 Transfer cells to a **37 °C** 5% CO_2 tissue culture incubator. Incubate for the appropriate time according to age of the culture:

- Early neuronal progenitors (~Day 11) - **00:10:00 (up to 20 mins)**
- Young mature neurons (~Day 30) - **00:20:00 (up to 30 mins)**
- Older mature neurons (~Day 52) - **00:25:00 (up to 35 mins)**

Check the progress of the dissociation after the indicated time. If necessary extend the incubation period for up to 10 mins more, checking the cells every few minutes.

7 For dissociation of mature neurons complete the preparation of **Wash Buffer 1** within the last 5 minutes of incubation of the cells.

- Retrieve the **DNase (D2)** vial(s) from **4 °C** storage.
- Reconstitute the vial(s) by adding 250 µL Wash Buffer 1 to each vial to make a 2mg/mL solution. Make sure to reconstitute all the powder (check the lid). Avoid vigorous mixing - do not vortex.
- Transfer the entire contents of the reconstituted vial(s) back into the Wash Buffer 1 tube. Use 250µL (1 vial) for every 15 mL buffer.

8 Following incubation inspect the cells under a microscope.

The cells should be detaching from the plate and the cell layer should have a darkened appearance. Cells will have a rounded appearance as they dissociate and individual cells should be visible at the edges or in gaps in the cell layer.

9 ***Optional:*** Test for ease of dissociation by gently pipetting ~ **100 µl** of the **Dissociation Buffer** against the cells with a P1000 pipette. If cells do not dissociate easily, extend digestion by **00:03:00** and repeat this step.

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10 Add **2 mL** of **Wash Buffer 1** per well.

11 ⚠

Use a P1000 pipette to repeatedly wash the buffer over the well(s) to detach the cells and dissociate them to a single-cell suspension.

Sufficient dissociation is critical at this step. It is recommended to check the cell suspension under a microscope and if necessary pipette the cell suspension up and down further until the majority is single cells. Try to avoid over-pipetting the cells as this could affect viability.

12 Transfer the cell suspension into a 15mL centrifuge tube capped with a 40µm cell strainer.

13 ***Optional:*** If there are residual cells in the plate, wash well(s) with **0.5 mL** of **Wash Buffer 1** and transfer this suspension into the tube from the previous step.

14 Centrifuge cells at **150 x g, Room temperature 00:03:00** .

15 Aspirate supernatant and gently re-suspend the cell pellet in **1 mL** of **Wash Buffer 2** with a P1000 pipette.

16 Transfer the cells to a Lo-bind 1.5mL Eppendorf tube.

We recommend preparing and thawing necessary reagents for the 10x loading procedure during these next steps if you have not already done so.

17 Repeat steps **14 & 15** three times in a mini centrifuge *(for a total of four centrifugations).*

Cell Counts

18 *Note: This step describes a 1 in 10 dilution for cell counting on a NucleoCounter®NC-200™. Depending on method of cell counting and number of cells, this dilution step may not be necessary.*

Prepare a cell count sample tube for each suspension prepared by adding **450 µl** of **Wash Buffer 2** to a fresh Lo-bind 1.5ml Eppendorf tube.

Take a **50 µl** aliquot of the well-mixed cell suspension and add this to the **450 µl** of **Wash Buffer 2** in the prepared counting tube(s).

19 Place the cell suspension tube(s) **On ice** during the cell counts and following steps.

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20 Count the cell suspension(s) on a NucleoCounter®NC-200™ and record % cell viability and viable cell concentration. Ensure less than 3% of cells are in aggregates.

If >3% of the cells are in aggregates, triturate the cell suspension by pipetting up and down with a P1000 pipette. Carry out a second cell count on a new sample. Alternatively, dilute the sample and pass through a cell strainer, then centrifuge and resuspend in 1mL Wash Buffer 2 before repeating the cell count.

21 Using the cell counts, calculate the dilution of the cell suspension(s) necessary to produce the desired cell number for loading for the 10x. Dilutions should be made using **Wash Buffer 2**, to a final volume of **1 mL** .

22 Keeping the cell suspensions **On ice** , proceed with the sample preparation and loading steps described in the 10x Genomics single cell gene expression kit guidelines.

23 We recommend performing a post-10x loading cell count on the diluted cell suspension. This cell count can normally be performed without further dilution (depending on the method of cell counting). This cell count will confirm the cell number loaded for 10x and check the cell viability has not dropped significantly since the first cell count. It can also check the percentage cell aggregates has remained acceptable.

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