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Regular non-enzymatic splitting of human pluripotent stem cells

Ralitsa RRM. Madsen[1]

[1]University of Edinburgh

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Ralitsa R. Madsen
University of Edinburgh, University of Cambridge

ABSTRACT

This protocol describes the non-enzymatic splitting of human pluripotent stem cells with ReLeSR (Stem Cell Technologies) and RevitaCell (Thermo Fisher Scientific). This method allows pluripotent stem cells to preserve their undifferentiated state and enhances their survival. Success will hinge upon:

- Ensuring the right size of colony clumps following ReLeSR treatment, which requires optimal pipetting (speed and number of mixing steps)
- Sticking to a consistent maintenance schedule (splitting the cells at regular intervals, preferrably at roughly the same time of day)

GUIDELINES

Abbreviations:

- E8/F: Essential 8 Flex
- E8/F + R: Essential 8 Flex supplemented with 1X RevitaCell
- DMEM/F12: Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham
- hPSCs: human pluripotent stem cells

MATERIALS

NAME	CATALOG #	VENDOR
1x DPBS	14190144	Gibco - Thermo Fischer
ReLeSR™ 500 mL	5873	Stemcell Technologies
Essential 8 Flex Medium Kit	A2858501	Thermo Fisher Scientific
Geltrex LDEV Free hESC Quality 5 ml	A1413302	Thermo Fisher Scientific
DMEM/F12 w/o L-Glutamine HEPES 500 ml	D6421-500ML	Sigma Aldrich
RevitaCell Suppplement 100X	A2644501	Thermo Fisher Scientific
Nunc™ Delta Cell-Culture Treated Multidishes; 6-well	140685	Thermo Fisher Scientific

MATERIALS TEXT

Other materials (all sterile if in direct contact with the cells):
Filter tips
Stripettes
Aspirator and aspirator tips
Benchtop microcentrifuge
Swinging bucket centrifuge
5 ml Eppendorf tubes
50 ml Falcon tubes

SAFETY WARNINGS

At all times, it is essential to be gentle when handling hPSCs and to limit their exposure to suboptimal environmental conditions.

BEFORE STARTING

Make sure to have

- Prepared Geltrex-coated plates in advice (see Geltrex coating protocol)
- Aliquots of E8/F with and without RevitaCell, equilibrated to room temperature

Preparatory work

45m

1 Pretreatment of the cells with E8/F+R (see Guidelines for abbreviations)

Ensure that the medium is fresh and has spent minimum time outside the fridge, thus limiting growth factor degradation. I typically make fresh aliquots from the stock medium in the fridge, thus keeping the stock at **4 °C** for most of the two weeks during which it can be used.

RevitaCell should be aliquotted into 500 µl aliquots (or other convenient volume) and stored at **-20 °C**.

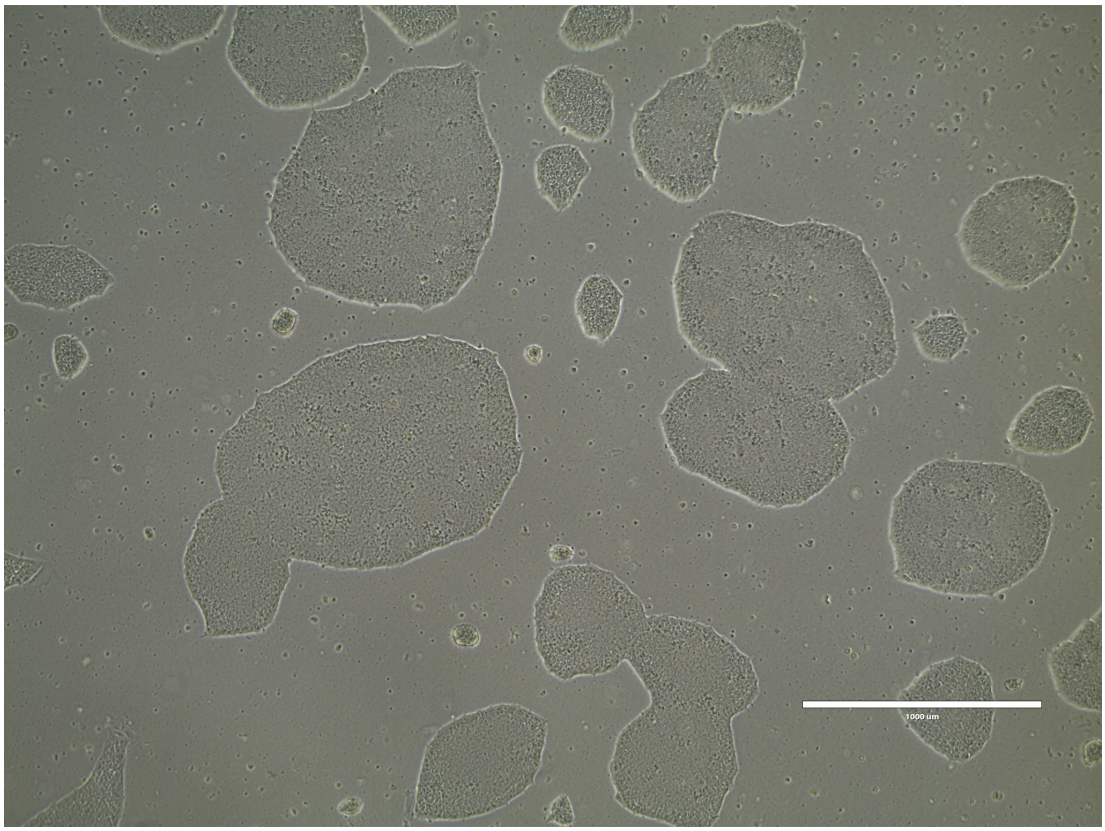
Once prepared, I usually keep aliquots of E8/F or E8/F+R for up to 3 days before discarding.

Keep track of all reagent lot numbers, particularly Geltrex and E8/F supplements!

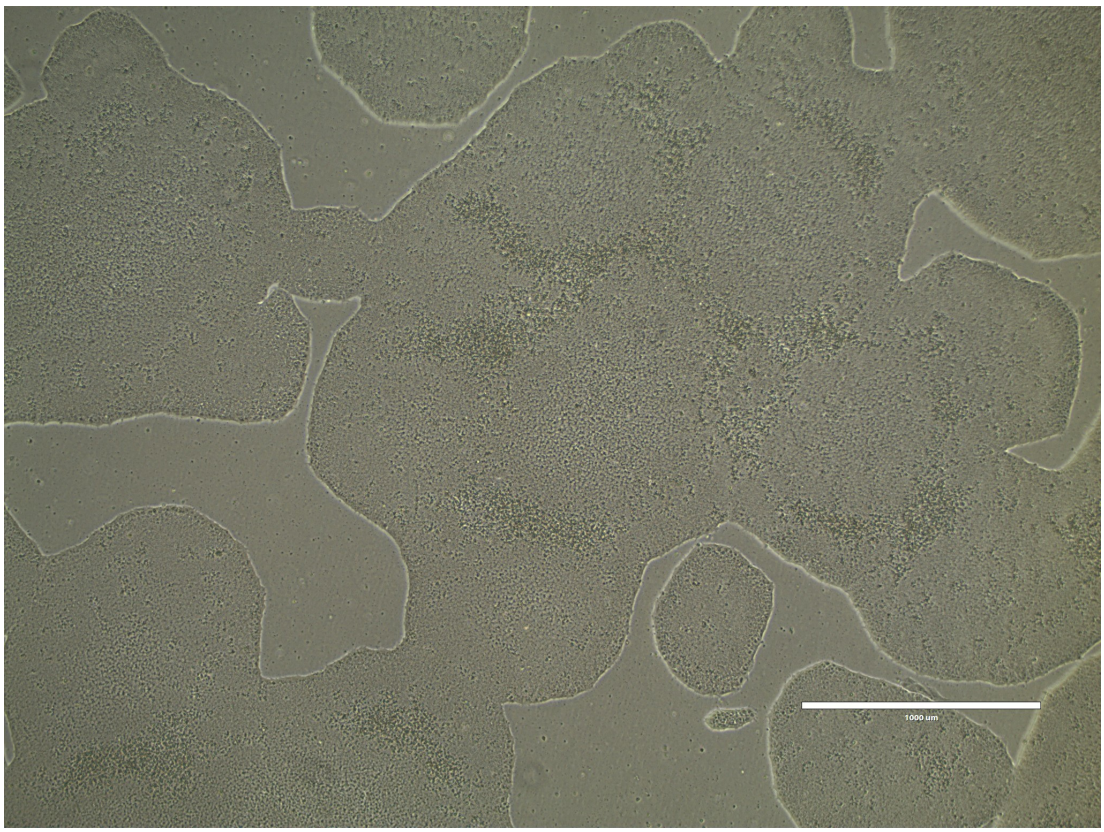
1.1 Dilute RevitaCell to 1X in E8/F and mix well. Leave to equilibrate to room temperature.

1.2 Equilibrate Geltrex-coated plates to room temperature (minimum **00:30:00** before intended use). For coating instructions, see the following protocol: Coating of plates with Geltrex, for human iPSC culture)

1.3 Inspect the cells to ensure that they exhibit the right morphological properties (high nucleus-to-cytoplasm ratio, tightly packed colonies with refractile edges, minimal/no differentiation) and are ready to be passaged (most colonies have diameter > 1000 µm; colonies merging/merged; relative confluence is 80-90 % thougn this might be cell line dependent).



Example image of an iPSC culture that is not ready to be split yet because most colonies are relatively small and have yet to start merging. Note, the healthy appearance of most colonies and the absence of differentiation. The same cultued 1 day later is shown below.



The same iPSC culture as on the previous image, but assessed 24 hours later. The culture is ready to be split.

1.4 Pretreat each 6-well of cells to split with 1.5 ml E8/F+R 30 min before processing (max 3 hours). The Revita enhances the survival of the

cells during subsequent processing steps, including attachment as small clumps at a relatively low density.

The pretreatment step may potentially be omitted - I have used it consistently with my cultures, partly because it was recommended in a CRISPR/Cas9 gene edting protocol.

Cell dissociation

15m

2 Inspect the Revita-pretreated cells under the microscope; you should notice that cells at the edges of the colonies have become more spiky - an effect of the Revita treatment as well as the medium exchange.

2.1 Transfer the 1.5 ml medium from each pretreated well to a 5 ml Eppendorf tube (I prefer this to 2 ml tube because it allows for better mixing subsequently and reduces the risk fo contamination).

The "pre-conditioned" medium has only been on the cells for a short period of time, so reusing it will save reagents. It is important, however, to ensure that all tubes/plates are well-labelled so conditioned media are not mixed across different cell lines.

It is absolutely essential that different cell lines are not cultured within the same 6-well dish. Apart from contamination issues, it is essential that the lines are split separately to minimise the amount of time individual cultures spend at suboptimal environmental conditions.

2.2 Gently rinse each well with 2 ml DPBS (add to the side of the well).

2.3 Remove the DPBS, add 1 ml ReLeSR to each well, leave for **00:01:00** and aspirate.

2.4 

Leave the cells at **37 °C** for **00:08:00** **(5-9 minutes)** .

This timing is cell line-dependent so will require checking - the cells are ready to be processed further when most colonies have loosened up, exhibiting refractory, white spaces between individual cells).

In the mean time, aspirate the excess Geltrex from the coated plates and add 1.5 ml of fresh E8/F+R to each well that will receive cells. Place in the incubator alongside the dissociating cells.

2.5 From the corresonding 5 ml tube with preconditioned E8/F+R medium (1.5 ml), take 1 ml and add to the dissociated cells, then hold tightly to the plate, place towards your body and tap against the plate (medium strength) for up to **00:01:00** to release the colonies. You should be able to see them coming off as larger clumps - if they are difficult to dissociate, that suggests insufficient incubation duration post-ReLeSR removal.

2.6 Using your P1000 pipette, rinse across each individual well, using the 1 ml medium that was added, in order to release any remaining colonies and to break up bigger clumps (2-3 times should be enough). Transfer the resulting suspension to the 5 ml Eppendorf tube containing the remaining 500 µl pre-conditioned medium, once again bringing the volume back to 1.5 ml.

Note, however, that pipetting has to be relatively gentle - any excess shear stress might compromise the health of your stem cell colonies.

2.7 ⚠

Close the 5 ml Eppendorf tube containing the cell suspension and look at the cells against a light source; if the suspension appears homogenous with clearly visible - but small - particles floating around without settling, the colony size is likely to be right and you can proceed with seeding. If the suspension is not homogenous and bigger clumps are seen to settle, pipette another 2-3 times to break the clumps up further.

Note that pipetting has to be relatively gentle - any excess shear stress might compromise the health of your stem cell colonies.

2.8 Seed the cells in the 6-well plate containing pre-warmed 1.5 ml medium in each well. The exact split ratio will be highly cell line-dependent, but it is essential that the cells are not seeded too densely to avoid differentation. Conversely, seeding them too sparsely will result in few survivals. In my experience, the optimum is between 1:10 and 1:15 (provided that you are using RevitaCell or another ROCK inhibitor). For example, I typically take 150 µl (for 1:10) or 100 µl (for 1:15) from the 1.5 ml cell suspension.

2.9 Inspect the cells under the microscope to ensure that the clumps are of the right size (50-200 µl). It is more critical to avoid big clumps than to avoid single cells because the RevitaCell will enhance single-cell survival. Therefore, if you see many big clumps, mix the suspension within each well gently again with the P1000 pipette (max 3 times).

Maintenance post-split

3 Roughly 24 hours following splitting, remove the RevitaCell by replacing the medium of the cells with 2 ml E8/F without RevitaCell. If seeded correctly, the colonies should appear healthy, yet fairly small and spaced apart, which means that they can skip feeding the following day (but only if you are using E8/F which contains a heat-resistance FGF2).

Feed the cells again 3 days post-split (2 ml E8/F).

The cells will usually be ready to split again 4 days post-split, but this will be cell line-dependent. For additional information and representative images, see step 3.7 in the following protocol: Thawing of pluripotent stem cells