

Troubleshooting feeder free cells

Problem	Possible Solutions/Precautions
Low viability after thaw <ul style="list-style-type: none"> • Little to no colonies visible within 4 days after recovery 	<ul style="list-style-type: none"> • Ensure you screen round the edges of the well, cells have a tendency to congregate at the sides of a well. • It is advisable to leave plates for a maximum of 2 weeks as colonies can appear within this time. This is not ideal for feeder free cultures. <p>To ensure quality of future vials:</p> <ul style="list-style-type: none"> • Ensure that cryovials are thawed quickly and that medium is added to the cells very slowly (drop-wise while gently swirling the tube). • Ensure that cells were banked at log phase of growth. • Try thawing cells into a smaller tissue culture vessel. • Ensure 10 µm ROCK inhibitor is added at thaw – if 24 hours post thaw nothing appears to have attached you can top up with 1 ml of medium plus 10 µm ROCK inhibitor, but after this point cells should be media changed daily and without ROCK inhibitor.
Low viability after passage <ul style="list-style-type: none"> • Cells do not attach properly • Non-typical morphology • High levels of cell death • Cells do not proliferate 	<ul style="list-style-type: none"> • Use lower split ratio and maintain a more confluent culture. • Ensure cells are in log phase of growth at passaging. • Avoid too long an incubation time in EDTA as cells might become more sensitive and colonies will disaggregate to single cells, which often die or differentiate when plated. • If cells do not come off easily Increase incubation time of EDTA. This is to avoid having to harshly rinse cells off thereby creating too small aggregates/ single cell suspension.
Non-uniform distribution of colonies within plate <ul style="list-style-type: none"> • Areas with too high density of iPS cells • In addition some areas may have fewer colonies 	<ul style="list-style-type: none"> • Make sure that the whole surface area of the tissue culture vessel is evenly coated with the appropriate matrix. • Ensure that the cell aggregates are evenly distributed by gently rocking the plate back and forth and side to side. Not in a circular motion. • Take care when placing plate into the incubator and leave undisturbed for 24h. • Ensure incubators are level and free from vibrations.
Colonies are not coming off the plate	<ul style="list-style-type: none"> • Ensure that incubation time and temperature of EDTA are in accordance with matrix. • Increase incubation time of EDTA without producing a single cell suspension. • Do not let cells become more than 80 % confluent. • Ensure the EDTA has not gone over its use by date or precipitated in the stock solution

<p>Cells detach after 1st medium change post passage.</p> <ul style="list-style-type: none"> Cells start to lift off even though they seemed to attach fine after passage 	<ul style="list-style-type: none"> Exchange medium very gently, do not drop medium rapidly onto the cells, tilt the plate and pipette medium down the side of the well. If colonies appear to be falling apart once cells compact then investigate the matrix, is it still in date, when were the plates made, were they made up correct, were they stored correctly, are the stocks being stored correctly.
<p>Spontaneous differentiation</p> <ul style="list-style-type: none"> Colonies do not have defined edges Cells within the colonies are less compact Cells appear flattened and bigger Morphology as depicted in C and D of the grading system (appendix 1) 	<p>In a bid to avoid in the first instance:</p> <ul style="list-style-type: none"> Ensure the plates used have coating that is not older than 7 days. Avoid leaving plates outside the incubator for more than 15 minutes. Ensure media is within date and has not been left in temperatures of room temperature or higher for extended periods (1-2 hours). iPSCs are extremely sensitive and any changes in reagent type or user-to-use technique variability can cause an increase in spontaneous differentiation. Fragment size at seeding can have an impact, too small or single cells, can spontaneous differentiate, too large and differentiation can form in the centre of the colony. <p>To tackle differentiation once it sets in:</p> <ul style="list-style-type: none"> Standard passaging is often the safest first option for 3-4 passages, especially if you are transitioning culture systems, as the cells need time to adapt. Passaging early i.e. before the dish has reached its usual confluence, as long as the iPSC colonies are compacted and of a reasonable size. Modifying the incubation time in EDTA, leaving it on slightly longer, replacing the EDTA with media and taping the plate as opposed to washing it in the hope that enough healthy colonies dislodge. This may require scale down of culture rather than usual split ratios. Manually scrapping the differentiation off the dish with a sterile tip. This can be hard to perform if you do not have a microscope in a hood, to get around this you can mark with a pen where the healthy colonies are and then take your plate into the hood and scrap off everywhere except the pen marks. Alternative to scrapping the differentiated cells off, you can colony pick the healthy colonies into a new 6 or 12 well dish. Split ratios as high as 1:20 can aid in removing differentiated cells, but you may risk karyotypic abnormalities especially if you persistently use high split ratios.

Troubleshooting feeder dependent cells

Problem	Possible Solutions/Precautions
<p>Low viability after thaw.</p> <ul style="list-style-type: none"> Little to no colonies visible within 2 weeks after recovery 	<ul style="list-style-type: none"> Ensure you screen round the edges of the well, cells have a tendency to congregate against the sides. Feeder dependants cultures can take up to 2 weeks to recover, you could potentially leave them for longer than this if nothing is identified on the dish but this is not ideal. <p>To ensure quality of future vials:</p> <ul style="list-style-type: none"> Ensure that cryovials are thawed quickly and that medium is added to the cells very slowly (drop-wise while gently swirling the tube). Ensure that cells were banked at log phase of growth. Try thawing cells into a smaller tissue culture vessel. Ensure 10 µm ROCK inhibitor is added at thaw and 24 hours post thaw top up with 1 ml of medium plus 10 µm ROCK inhibitor, but after this point cells should be media changed daily and without ROCK inhibitor. Try and avoid dissociating thawed colonies to single cells when plating. Ensure the confluence and health of the MEFs is adequate. If necessary top up the MEFs, this can occur even with iPSCs on the dish but take care to not over seed the MEFs.
<p>Low viability after passage</p> <ul style="list-style-type: none"> Cells do not attach properly Non-typical morphology High levels of cell death Cells do not proliferate 	<ul style="list-style-type: none"> Ensure cells are in log phase of growth at passaging. Avoid leaving collagenase and dispase on for too long (don't exceed 1 hour), as cells will dissociate to single cells which won't survive the passage. Ensure the confluence and health of the MEFs is adequate.
<p>Non-uniform distribution of colonies within plate</p> <ul style="list-style-type: none"> Areas with too high density of iPS cells In addition some areas may have fewer colonies 	<ul style="list-style-type: none"> Make sure that MEFs are evenly distributed across the dish at sufficient confluence. Ensure that the cell aggregates are evenly distributed by gently rocking the plate back and forth and side to side. Not in a circular motion. Take care when placing plate into the incubator and leave undisturbed for 24h. Ensure incubators are level and free from vibrations.
<p>Colonies are not coming off the plate post 45 minute incubation in collagenase and dispase</p>	<ul style="list-style-type: none"> Do not allow colonies to become too large and dense. Do not let cells become more than 80 % confluent. Extend incubation of collagenase and dispase from 45 minutes to 1 hour (do not exceed 1 hour). After a 1 hour incubation take what you can, but remember to scale back the surface area you seed over in accordance with what you obtained from the original dish. Ensure the collagenase and dispase is between room temperature and 37°C before use.

	<ul style="list-style-type: none"> • Ensure the collagenase and dispase has not become inactive over time due to thermal cycling.
Centre of colonies become over grown <ul style="list-style-type: none"> • Darker discolouration in the centre • Centres potentially detach, leaving 'donut' colonies <p>This can lead to differentiation in the centre of the colonies</p>	<ul style="list-style-type: none"> • Passage when the individual colonies are of a reasonable size and clearly compacted, don't wait for the whole surface to be of a reasonable confluence.
Spontaneous differentiation <ul style="list-style-type: none"> • Colonies do not have defined edges • Cells within the colonies are less compact • Cells appear flattened and bigger • Morphology as depicted in C and D of the grading system (appendix 2) 	<p>In a bid to avoid in the first instance:</p> <ul style="list-style-type: none"> • Ensure the plates used have healthy MEFs. • Avoid leaving plates outside the incubator for more than 15 minutes. • Ensure media is within date and has not been left in temperatures of room temperature or higher for extended periods (1-2 hours). • iPSCs are extremely sensitive and any changes in reagent type or user-to-use technique variability can cause an increase in spontaneous differentiation. • Fragment size at seeding can have an impact, too small or single cells, can spontaneous differentiate, too large and differentiation can form in the centre of the colony. • Don't allow individual colonies to over grow or become too compact in the centre. <p>To tackle differentiation once it sets in:</p> <ul style="list-style-type: none"> • Standard passaging is often the safest first option for 3-4 passages, especially if you are transitioning culture systems, as the cells need time to adapt. • Passaging early i.e. before the dish has reach its usual confluence, as long as the iPSC colonies are compacted and of a reasonable size. • Be more gentle with the dish post collagenase and dispase incubation in a bid to just take healthy colonies and leave the single cells

Appendix 1 – Feeder Free Grading System

<p>Morphology grade</p> <p>A</p> <ul style="list-style-type: none"> + Well-rounded colonies + Smooth, defined edges + Compacted cells. + May see slightly uneven/speckled colony surface (stippling-type effect), mostly due to overgrowth + Minimum or very low levels of overgrowth <p>Differentiation: None - Low.</p> <p>Feeder-free</p>	
<p>Morphology grade</p> <p>B</p> <ul style="list-style-type: none"> + Well-rounded colonies + Most colonies have smooth, defined edges + Mostly compacted cells + Overgrowth /differentiation that has led to some ring-shaped or 'egg' like colonies + Rescuable <p>Differentiation: Low - Medium</p> <p>Feeder-free</p>	

Morphology grade

D

+ Irregularly shaped colonies without defined edges
 + Few compacted cells
 + No obvious/very few areas of compacted cells
 + Only rescue-able colonies remaining are completely uncompacted
 + Very difficult/unable to rescue

Differentiation level:
High

Feeder-free

Morphology grade

C

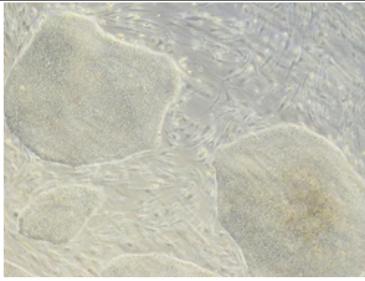
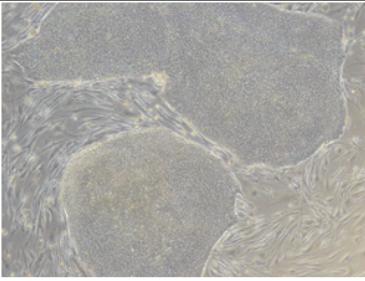
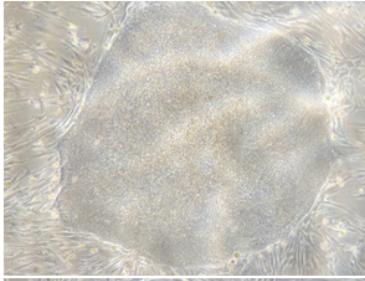
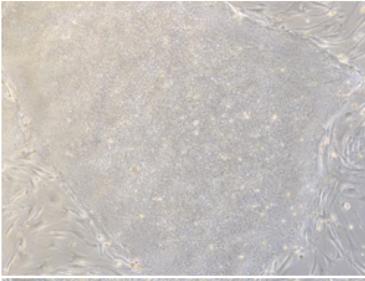
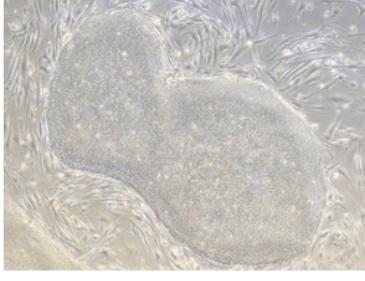
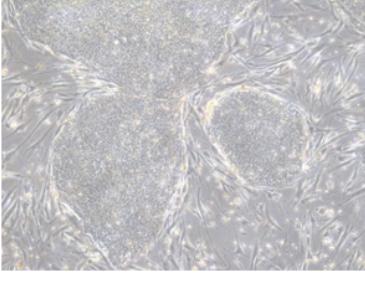
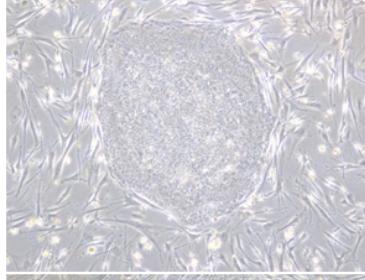
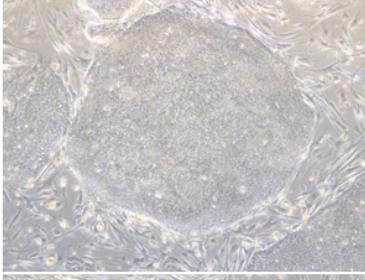
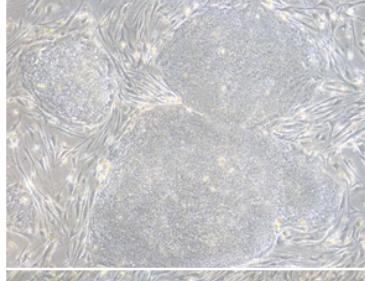
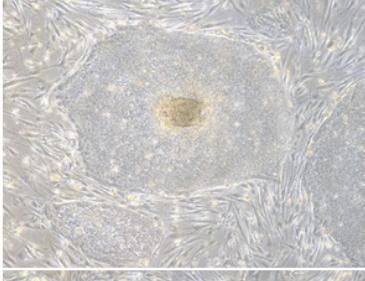
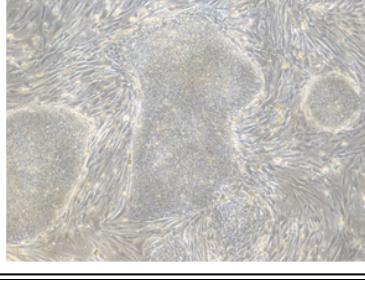
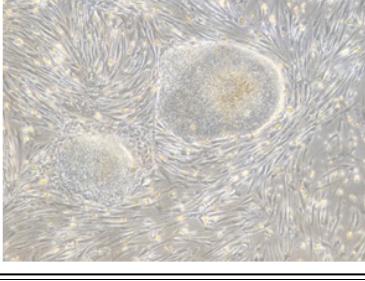
+ Well-rounded colonies with defined edges
 + Most colonies have smooth, defined edges, some irregularly shaped colonies
 + Majority compacted cells with some overgrowth or differentiation present at edges of or outside colonies, may well surround colonies.
 + Rescuable

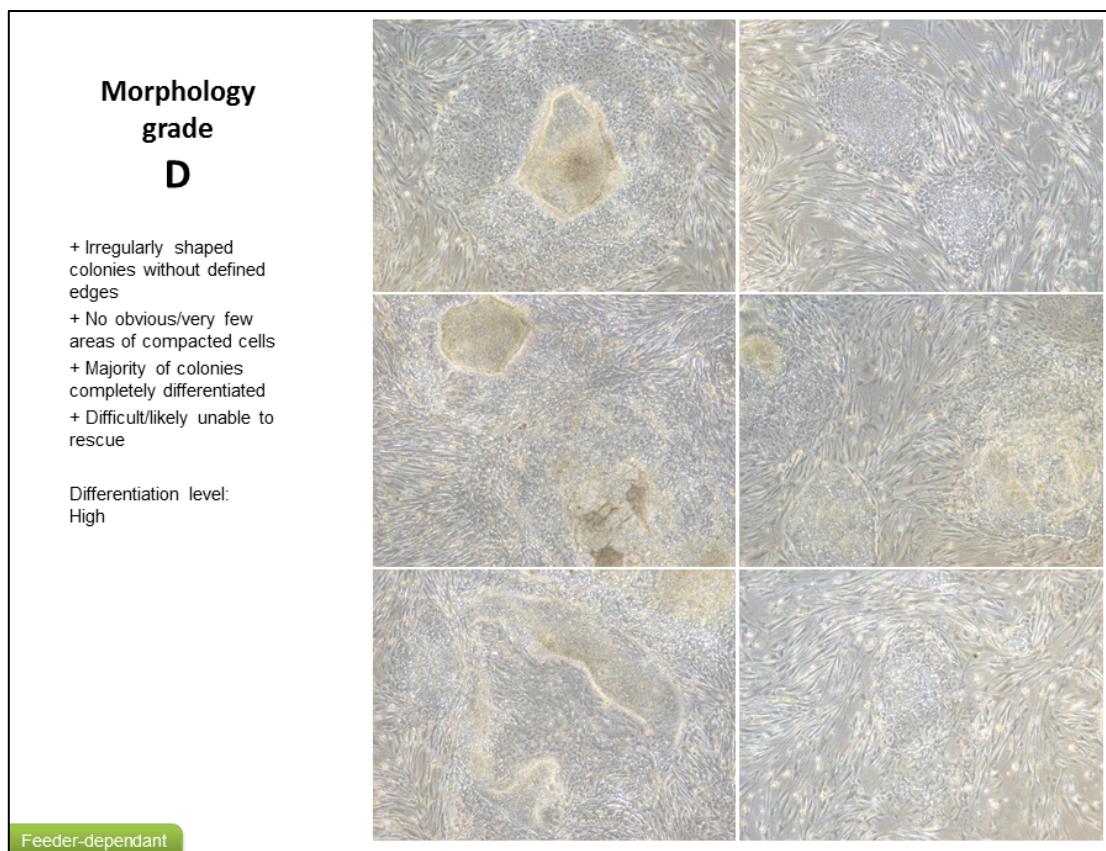
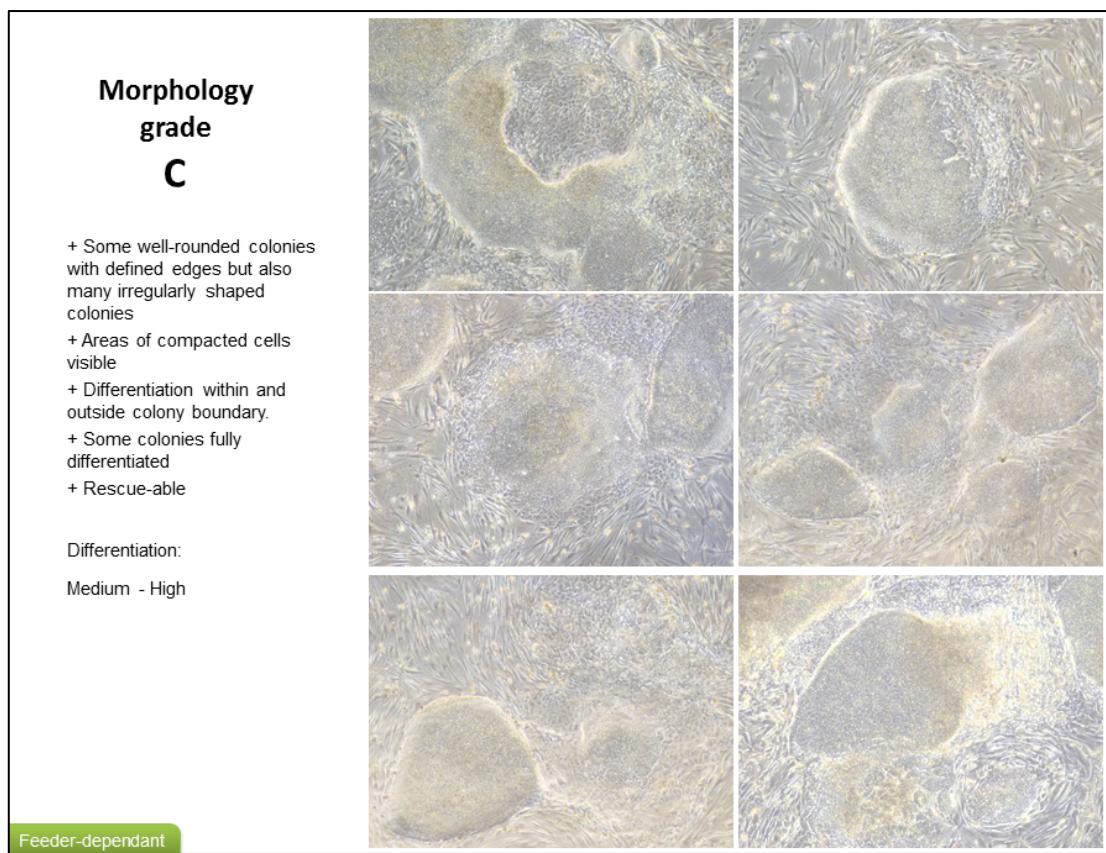
Differentiation:
Medium - High

N.B. Some colonies may look uncompacted. To recover, these will need passaging sooner.

Feeder-free

APPENDIX 2 – FEEDER DEPENDANT GRADING SYSTEM

<p>Morphology grade</p> <p>A</p> <ul style="list-style-type: none"> + Well-rounded colonies + Smooth, defined edges + Compacted cells + May see slightly uneven/speckled colony surface (stippling-type effect) + Minimum or very low levels of overgrowth <p>Differentiation: None - Low.</p>	     
<p>Morphology grade</p> <p>B</p> <ul style="list-style-type: none"> + Well-rounded colonies + Most colonies have smooth, defined edges + Compacted cells with some overgrowth or slightly uneven colony surface (stippling-type effect) + Differentiation present at edges of or outside colonies <p>Differentiation: Low - Medium</p>	     



Many thanks to Lucy Weston-Stiff for producing