Version 1 2016

Allen Institute for Cell Science

Manual Plating of WTC Cells on Glass Bottom Imaging Plates for Imaging Purposes

Required reagents

- Complete, phenol red mTeSR1 culture media, referred to in this protocol as simply "mTeSR1" 400 mL basal media with provided 100 mL 5x supplement (Stem Cell Technologies #05850) with added 1% penicillin streptomycin (Gibco #15070-063)
  Warm mTeSR1 media at room temperature only! Refer to page 16 of the Stem Cell Technologies technical manual about preparation, storage and shelf life of this media.
- Complete, phenol red-free mTeSR1 culture media, referred to in this protocol as simply "phenol red-free mTeSR1": 400 mL basal media with provided 100 mL 5x supplement (Stem Cell Technologies custom order) with added 1% penicillin streptomycin (Gibco #15070-063)
  Warm phenol red-free mTeSR1 media at room temperature only! Refer to page 16 of the Stem Cell Technologies technical manual about preparation, storage and shelf life of this media.
- Phenol red-free Matrigel (Corning #356231)
- Phenol red-free DMEM/F12 (Gibco #11330-032)
- 10 mM Rock inhibitor (Y-27632, Stemgent #04-0012-10) reconstituted in DMSO (Sigma #D2650) as per manufacturer's instructions.
- DPBS without $Mg2^{+}$ or Ca^{2+} (Gibco #14190-144)
- StemPro Accutase (Gibco #A11105-01)
- 10 cm tissue culture treated dishes (Corning #353003) or 6 cm tissue culture treated dishes (Corning # 353002)
- 24-well glass bottom plate with high performance #1.5 cover glass (CellVis # P24-1.5H-N) or 96-well glass bottom plate with high performance #1.5 cover glass (CellVis #P96-1.5H-N).

We tested several glass bottom imaging plates. In our hands, the WTC cells plated best on CellVis glass bottom plates.

All work described in this protocol should be performed in a sterile Bio Safety Cabinet (Nuaire Class II Type 2A or similar) using proper sterile technique.

Aliquot 10 mL phenol red-free Matrigel vial into single use aliquots

(1) Phenol red-free Matrigel is stored long term at -80ºC. Thaw the 10 mL glass vial overnight at **4ºC**.
Do not thaw Matrigel at room temperature or in 37ºC water bath as Matrigel polymerizes above 10ºC.
(2) Pre-chill 15 mL conical tubes or 1.5 mL centrifuge tubes on ice or at -20ºC.
(3) Aliquot the thawed phenol red-free Matrigel into the conical tubes or 1.5 mL centrifuge tubes **on ice** (see Table 1 for commonly used aliquot sizes). Work carefully, yet as fast as possible.
(4) Store aliquots at -20ºC short term for up to 3 months.

Manual coating of glass bottom imaging plates with phenol red-free Matrigel

(1) Thaw desired aliquot amount of phenol red-free Matrigel based on plate format and number of wells to be imaged (please refer to tables 1 and 2) at **4ºC**. Make sure the Matrigel is completely thawed. This might take between 30 minutes and two hours depending on the aliquot size.
(2) Prepare mTeSR1 + Rock inhibitor media. Add Rock inhibitor at 1000X dilution to room temperature mTeSR1 media and mix well by pipetting up and down.
(3) Dilute thawed phenol red-free Matrigel 1:30 with **4ºC** phenol red-free DMEM/F12 **on ice (0-4ºC)** (see Table 1 for commonly used volumes). Gently mix solution by pipetting the entire volume up and down at least **five times on ice**.
Inadequate mixing will cause uneven polymerization of the Matrigel, resulting in the presence of aggregates and/or auto-fluorescent precipitates in the Matrigel coating.
(4) Diluted phenol red-free Matrigel should be used immediately and should not be re-frozen. Add diluted Matrigel to the bottom of each well as specified in Table 2. Make sure diluted Matrigel is distributed over the entire bottom of the well. Gently tap corners of imaging plate to distribute diluted Matrigel in the wells if needed. Keep the working stock of diluted Matrigel **on ice**.
(5) Incubate the phenol red-free Matrigel-coated imaging plate **at least one hour** and up to 1.5 hours **at room temperature**.
Longer incubation times at room temperature or storage of Matrigel-coated imaging plates at 4ºC leads to the formation of auto-fluorescent precipitates in the Matrigel. Never store Matrigel-coated imaging plates for imaging at 4ºC.
(6) Slightly tilt the imaging plate and aspirate and discard **all** excess diluted phenol red-free Matrigel. To avoid drying of wells, ensure that all Matrigel-to-media exchange is done within 30 seconds. We usually seed cells immediately after removal of excess Matrigel as

described below in “seeding cells”. In case cells are not ready to be seeded after Matrigel is removed, gently add 50-100 µL (96 well plate) or 250-500 µL (24-well plate) room temperature mTeSR1 + Rock inhibitor to the well. Make sure that the entire bottom of the well is covered with media. Store glass bottom plate in the tissue culture incubator (37ºC, 5% CO_2) if cells will not be seeded within 10 minutes of adding media. Seed cells within one hour of adding media to wells.

Table 1. Single-use phenol red-free Matrigel aliquots and the required DMEM/F12 volumes to add for 1:30 dilution

undiluted Matrigel (µL)	DMEM/F12 (µL)	final volume 1:30 diluted Matrigel (µL)
3.33	96.67	100
33.33	966.67	1000
66.66	1033.34	2000
133.33	3866.67	4000
266.67	7733.33	8000

Table 2. Amounts of diluted phenol red-free Matrigel required for optimal coating of one well of 24-well and 96-well imaging plates

type of imaging plate	1:30 diluted Matrigel (µL)
24-well	500
96-well	100

Seeding cells

(1) Passage cells according to “Culture and freezing methods for WTC” until the point that cells are in suspension and counted.
We usually use cells grown on 10cm or 6 cm tissue-culture treated dishes as source cells when plating WTC cells on glass bottom imaging plates.

(2) Mix the cells by pipetting the entire cell suspension up and down three to five times. Cells settle quickly in suspension, so suspension should be mixed every 2-3 minutes. Dilute cells as specified in Table 3 for seeding cells onto the imaging plates, mix and add to the phenol red-free Matrigel-coated wells from step (6) in the section above. For even distribution of the cells in the wells, avoid tilting the plate during and after cell seeding until cells have adhered to the Matrigel (2-3 minutes). If seeding cells in 96-well imaging plate, gently tap two adjacent corners of the plate to evenly distribute the cells in the well. If seeding cells in 24 well imaging plates, gently move plate back and forth and right to left or tap two adjacent corners of the plate to evenly distribute cells in well.

Avoid adding a small volume of concentrated cell suspension to each well. Instead, dilute cell suspension to a desired concentration and plate a larger volume, preferably the final plating volume.

Table 3. Optimal seeding densities of cells in 24 well and 96 well imaging plates to maintain mature colonies three and four days after seeding

type of imaging plate	final plating volume	cell number for day 3	cell number for day 4
24 well	1000 µL	12500	12500
96 well	150-200 µL	2500-3000	2000-2500

Maintaining cells in glass bottom plates until imaging

(1) Feed cells daily with room temperature mTeSR1 media with the amount specified in Table 3 under final plating volume. Switch to phenol red-free mTeSR1 media the day before imaging. We usually do not feed the cells at the day of imaging unless specified by our experimental protocols. If cells have to be fed or washed at the same day as they are being imaged, we use phenol red-free mTeSR, which was pre-equilibrated in the tissue culture incubator (37 ºC, 5% CO_2) one hour to overnight.

(2) If experimental procedure requires washing of the cells prior to imaging, pre-equilibrate complete, phenol red-free mTeSR1 in the tissue culture incubator (37ºC, 5% CO_2) at least one hour to overnight before using it to wash cells.

Imaging

Different colony and cellular morphologies can usually be observed, but healthy, mature and immature colonies should be predominant. The ratio of mature to immature colonies might vary depending on the length of time since seeding and the growth rates of individual cell lines. We usually aim to image mostly mature and healthy colonies for most of our daily imaging purposes. Example images were taken at 10x magnification unless specified otherwise.

Mature and healthy colonies

Mature colonies are sheet-like with smooth edges. The majority of cells should be tightly packed in mature colonies (colonies A- E). However, colonies with fewer tightly packed cells may be classified as mature if the remainder of cells in the colony are not too loosely packed (colony F). For example, both colony F and M contain some tightly packed cells, but the width and depth of fissures between the cells that are not well

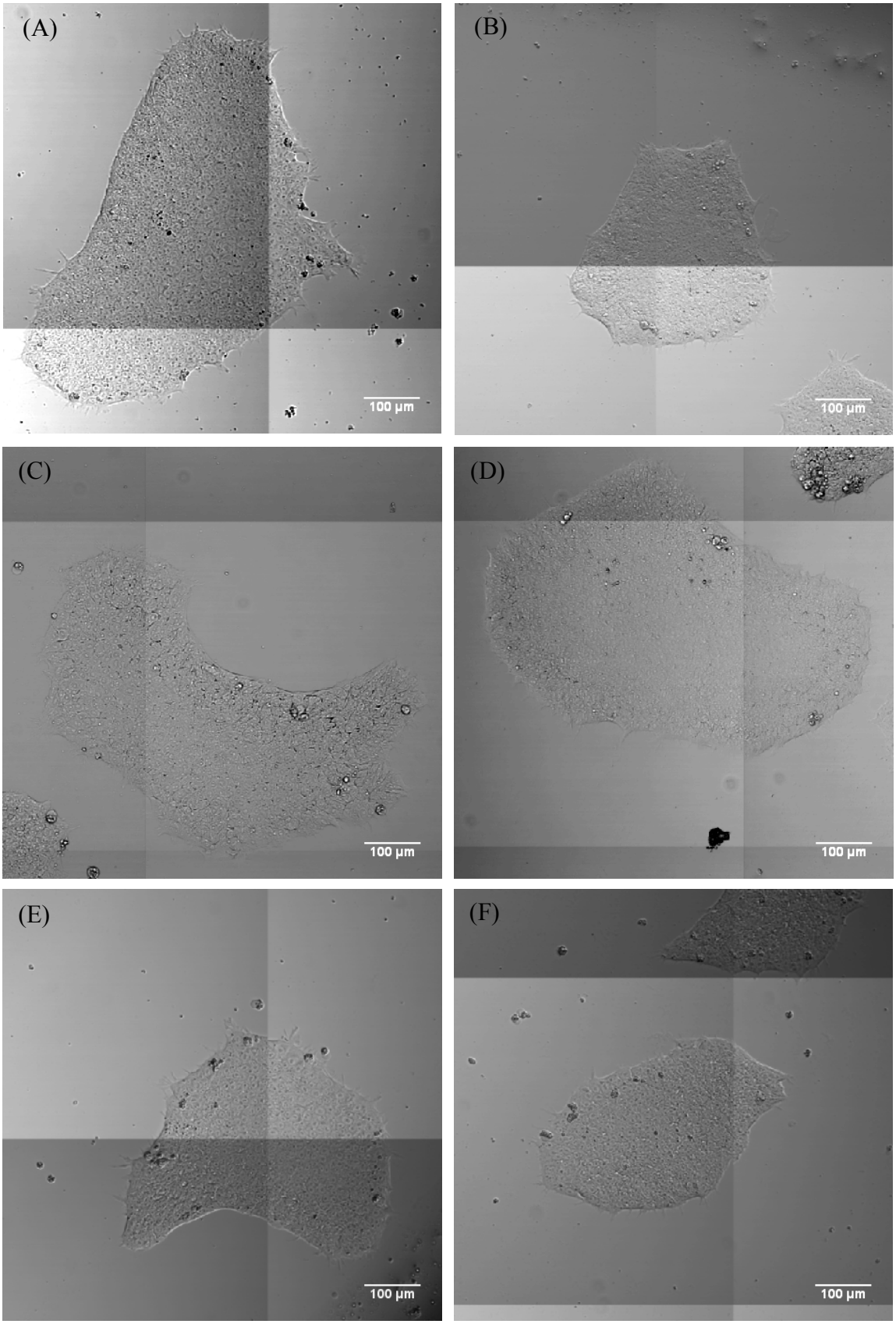
packed are smaller in colony F compared to those in colony M. Therefore, colonies like colony F could be considered mature while colonies like colony M could be considered immature. Colonies can have highly curved edges (colonies C and E), which seems to be a more glass-plating specific morphology. Sometimes these colonies have less tightly packed and taller cells in their curved region (colony C), which we are usually trying to avoid imaging. Colonies G and H are examples of mature colonies at 100x magnification.

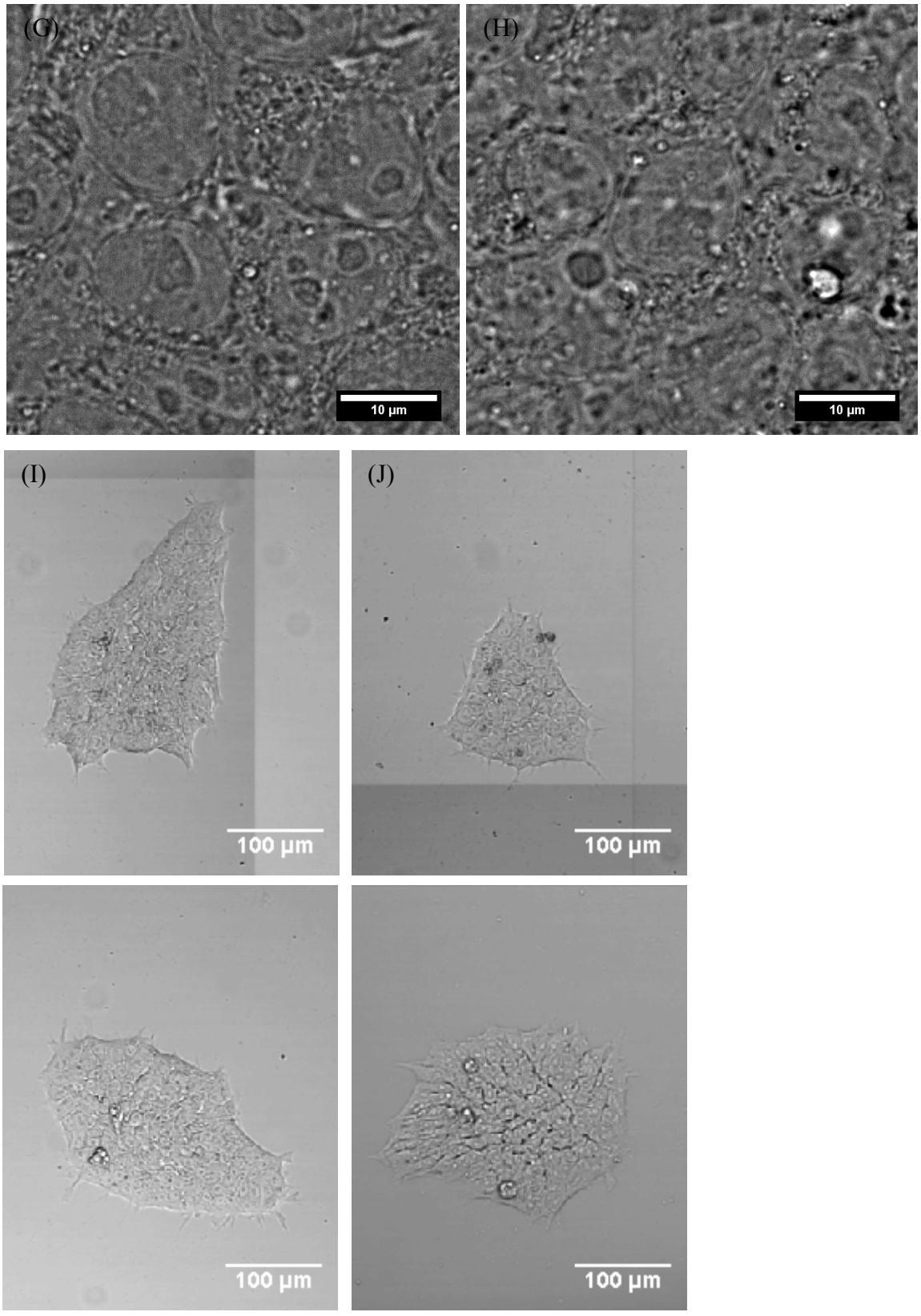
Immature and healthy colonies

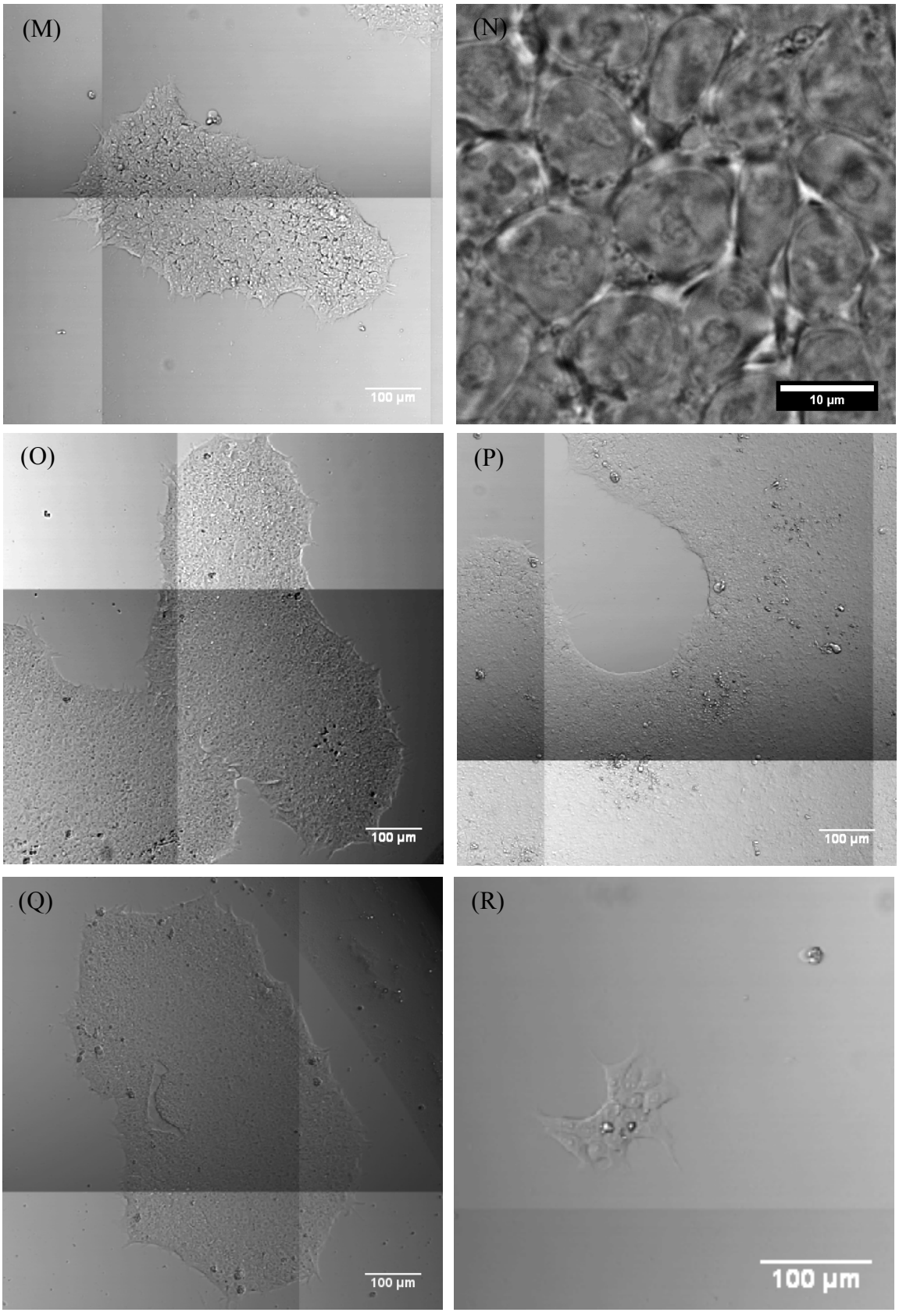
Immature colonies are characterized by loosely packed cells with wide and deep fissures between cells (colonies I-M). Colony N is an example for an immature colony at 100x magnification.

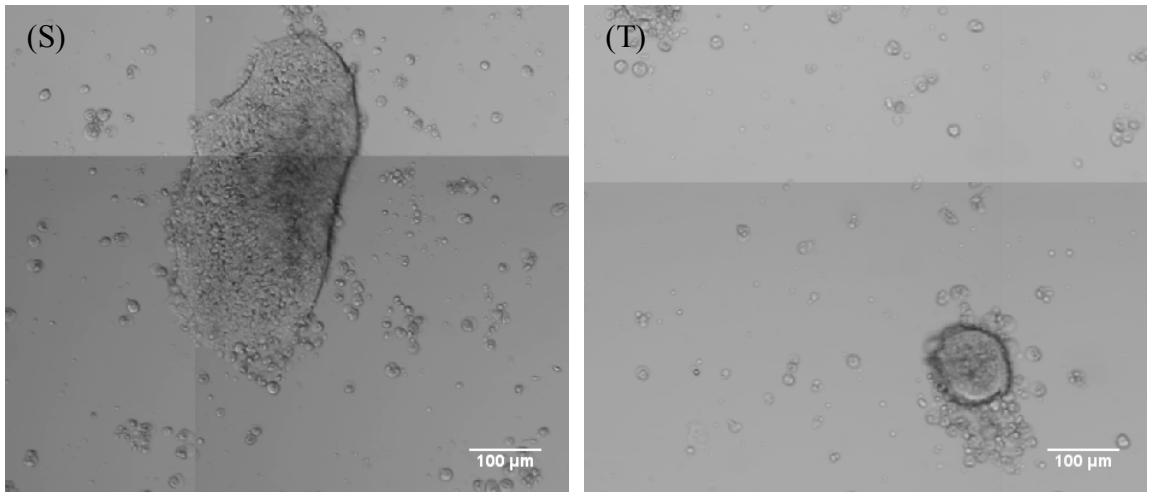
Other colony or cell morphologies, which can be observed in addition to mature and immature colonies

Areas of fusion between two or more colonies normally differ in their degree of organization and packing compared to mature colonies (colony O). Over-mature colonies can contain regions of cell overcrowding in which increased cell death is typically observed (colony P), or there might be a crater forming within the colony (colony Q). Colonies containing differentiating cells are characterized by the presence of flat and well-spread cells (colony R). Colonies that are not well attached to the Matrigel or are dying might lift off partially (colony S) or nearly completely (colony T) from the substrate with the latter leading to the formation of balled up colonies that are not suitable for imaging.


(A)
100 µm
(B)
100 µm
(C)
100 µm
(D)
100 µm
(E)
100 µm
(F)
100 µm


(G)
10 µm
(H)
10 µm
(I)
100 µm
(J)
100 µm
100 µm
100 µm


(M)
100 µm
(N)
10 µm
(O)
100 µm
(P)
100 µm
(Q)
100 µm
(R)
100 µm


(S)
100 µm
(T)
100 µm