molecular probes
life technologies

Improving the stem cell reprogramming characterization workflow: *better colony identification using live-cell reagents that enable simultaneous detection of positive and negative pluripotency markers*

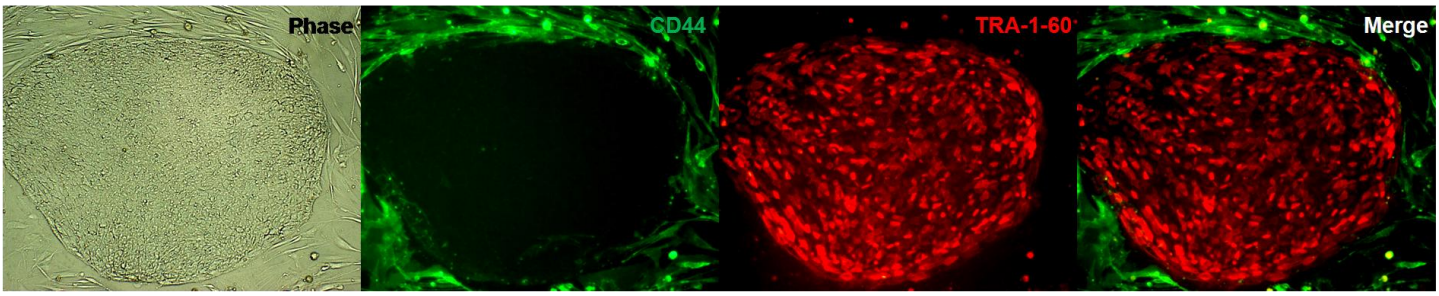

Figure 1 – iPSC colony growing on a MEF feeder layer co-stained with antibodies against CD44 (green) and TRA-1-60 (red).

One of the most important steps during a typical stem cell reprogramming workflow involves the identification and picking of a set of candidate colonies for further culture expansion and detailed characterization in order to obtain high quality induced pluripotent stem cell (iPSC) lines. Once reprogramming is initiated, the cell transformation process can take up to several weeks to come to completion for just a fraction of the original set of primed cells and their progeny. As a result, these cultures end up as a highly heterogeneous mixture of non-reprogrammed, "partially" reprogrammed and "fully" reprogrammed cells. In addition, these cultures are commonly grown in the presence of a feeder cell layer which adds to the complexity of reprogramming cultures. Not surprisingly, tools that can facilitate better and/or earlier detection of fully reprogrammed stem cell colonies are in demand.

Traditional approaches to iPSC colony identification have largely relied upon careful observation of the morphology changes that take place during reprogramming. For instance, elongated fibroblasts subjected to reprogramming protocols gradually transform into rounded cells with a propensity to form three-dimensional colonies. Cell clustering may occur as early as 7 days after initiating reprogramming. However, the majority of these cells will not yet be fully reprogrammed. By approximately day 14, emerging iPSC colonies start to become more apparent, including groupings of compact, flattened-cells with high nucleus to cytoplasm ratios and clearly visible boundaries that gives rise to a distinctive cobblestone-like colony appearance. By days 21 - 28, the colonies are usually large enough to be transferred to new culture dishes.

Ideally, each of the colonies picked for further culture and analysis contains only fully reprogrammed or bona fide pluripotent cells. However, in practice the colonies that emerge usually consist of varying degrees of partially and fully reprogrammed cells, resulting in a hodgepodge of cells that exhibit subtle to almost indistinguishable morphology differences amongst them even to the well-trained eye. Since it can be difficult to clearly distinguish fully reprogrammed colonies using morphology alone, a common practice is to pick not just a few, but multiple colonies to carry forward in order to increase the chance of successfully obtaining a high quality iPSC line in the end. While generally effective, this numbers approach to colony selection is laborious, costly and time-consuming.

Here we illustrate how live-cell immunofluorescence staining for positive and negative cell surface markers of pluripotency offers several advantages over traditional morphology observations when monitoring the reprogramming process and deciding which colonies to pick. TRA-1-60 is commonly used as a positive cell surface marker for pluripotent stem cell identification (**Figure 1**). CD44, which is found on many differentiated cell types including fibroblasts but not pluripotent stem cells, can be used as a negative marker. The combination of both markers is useful for identifying fully reprogrammed iPSC colonies (**Figures 2 – 6**).

The live-cell staining approach to monitoring the presence of key stem cell markers is embodied in the Molecular Probes® stem cell kits for live-cell imaging (**Table 1**). These kits pair together Alexa Fluor® dye-conjugated antibodies against cell surface markers with a superior imaging medium, FluoroBrite™ DMEM, which is designed to maximize fluorescence signal-to-background and preserve cell health during live-cell imaging workflows. These reagents are specially formulated and sterile-filtered to meet the demands of live-cell imaging applications: they contain no cytotoxic preservatives (e.g., no sodium azide) and are tested to ensure that endotoxin levels are low and that they are free of common microbial contaminants.

Table 1 – Description of the Molecular Probes® human pluripotent stem cell live imaging kits.

Cat. No.	Product name	Description
A25528	CD44 Rat anti-human/mouse mAb, AlexaFluor® 488 Conjugate Kit for Live Cell Imaging	CD44 is a cell surface glycoprotein that is present on many differentiated cell types including fibroblasts. In addition to marking fibroblasts (e.g., stem cell feeder layers or non-reprogrammed fibroblasts), CD44 is a useful negative stem cell marker during reprogramming since its expression is lost as cells become pluripotent (also see Quintanilla et al. [2014] PLoS ONE e85419*).
A25618	TRA-1-60 Mouse anti-human mAb, AlexaFluor® 488 Conjugate Kit for Live Cell Imaging	TRA-1-60 antigen is a widely used positive stem cell marker. It has been associated with certain carbohydrate modifications present on the cell surface glycoprotein podocalyxin. TRA-1-60 antigenic sites are found on pluripotent stem cells but are lost when these cells undergo differentiation.
A24879	TRA-1-60 Mouse anti-human mAb, Alexa Fluor® 555 Conjugate Kit for Live Cell Imaging	
A24882	TRA-1-60 Mouse anti-human mAb, Alexa Fluor® 594 Conjugate Kit for Live Cell Imaging	

*http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0085419

Reference protocol for live-cell immunofluorescence staining

For a complete protocol, please see the following link:
http://tools.lifetechnologies.com/content/sfs/manuals/stemcell_ab_kits_live_cell_imaging_man.pdf

1. Centrifuge the dye-conjugated antibody solution (e.g., 2 minutes at 10,000 x g) and only use the supernatant.

   This step eliminates any antibody aggregates that may have formed during storage, thereby reducing non-specific background staining.

2. Add a 1:50 volume of the dye-conjugated antibody directly to the cell culture medium of the cells to be stained. Mix by gentle swirling.

3. Incubate for 30 minutes at 37°C.

4. Remove the staining solution and gently wash the cells 2 – 3 times with FluoroBrite™ DMEM (Cat. No. A1896701).

5. For optimal results, image the cells immediately (i.e., within 30 minutes).

   To continue culturing the cells, simply replace the FluoroBrite™ DMEM with fresh cell culture medium and return the cells to the 37°C incubator.

Notes

The images used in figures 1 – 6 were acquired on an EVOS® FL Imaging System (Cat. No. AMF4300).
For stem cell reprogramming, we recommend the CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Cat. No. A1378001).

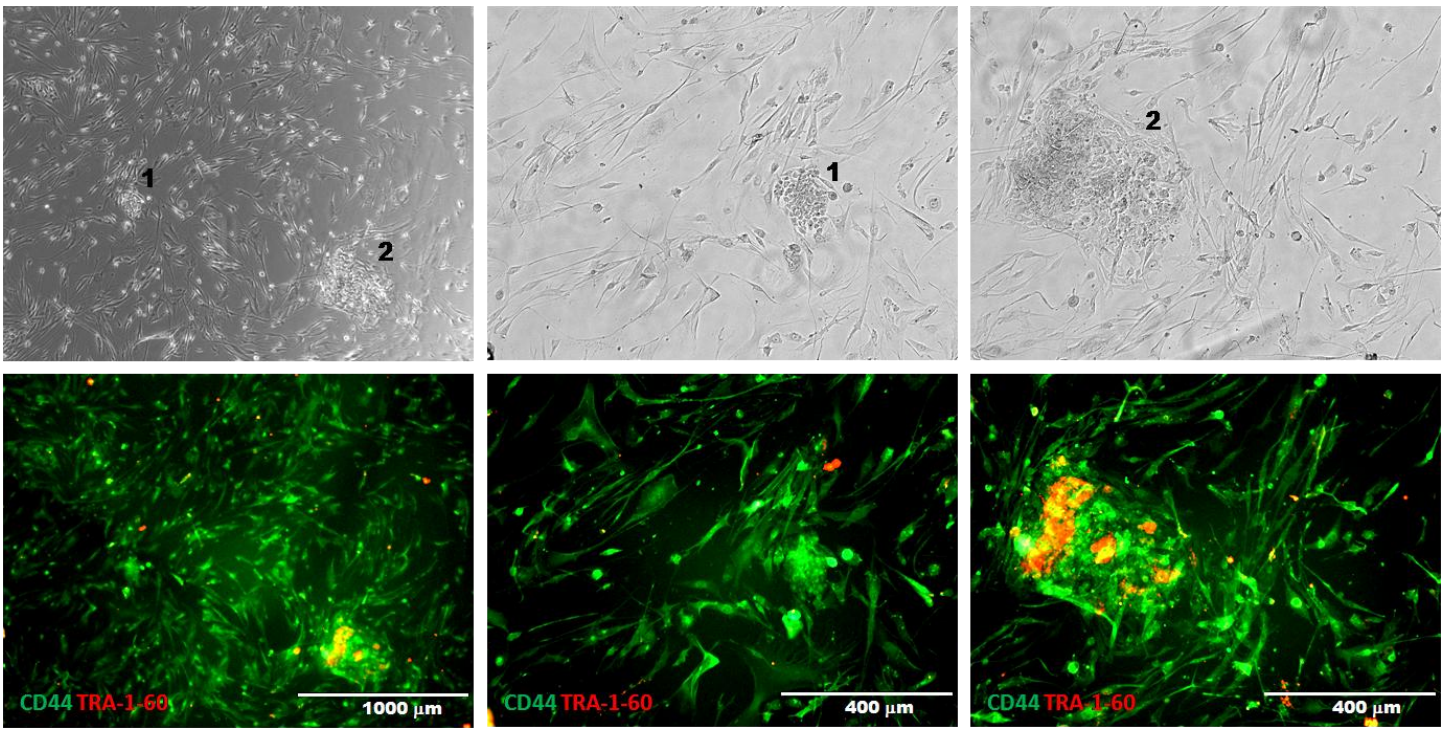

Figure 2 – At day 11 after initiating reprogramming of BJ fibroblasts, a culture growing on a MEF feeder layer was co-stained with live-cell qualified antibodies for CD44 (green, negative pluripotency marker that also stains the feeder cells) and TRA-1-60 (red, positive pluripotency marker). Phase and fluorescence images were taken at lower magnification (left panel), with two emerging colonies detected, or at higher magnification (middle and right panels) to view the individual colonies. The immunofluorescence staining indicates that colony #1 (CD44 positive / TRA-1-60 negative) consists of non-reprogrammed / non-pluripotent cells whereas colony #2 is "partially" reprogrammed (mixture of CD44 and TRA-1-60 positive cells).

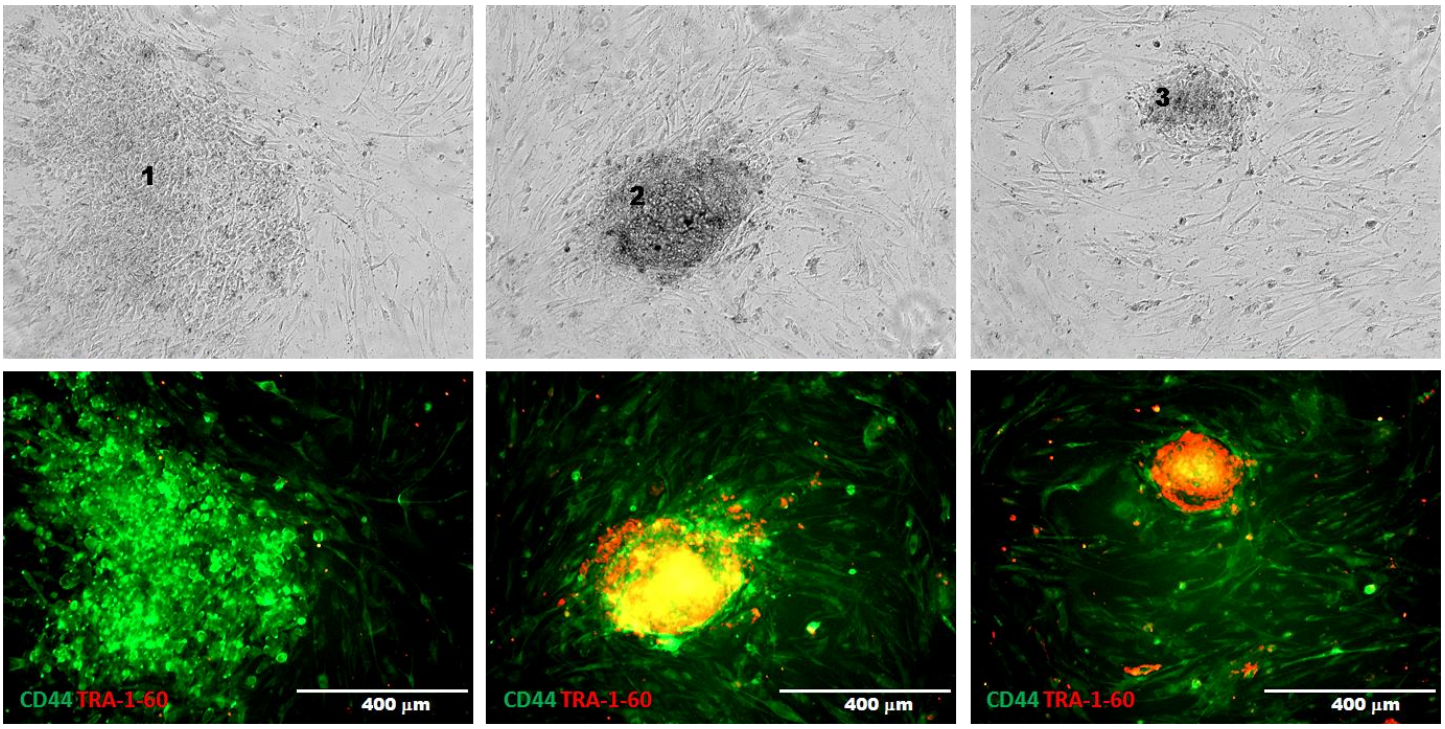

Figure 3 – At day 17 after initiating reprogramming of BJ fibroblasts, a culture growing on a MEF feeder layer was co-stained with live-cell qualified antibodies for CD44 (green, negative pluripotency marker that also stains the feeder cells) and TRA-1-60 (red, positive pluripotency marker). Phase and fluorescence images were taken of three distinct colonies: colony #1 (left panel) consists of non-reprogrammed / non-pluripotent cells (CD44 positive / TRA-1-60 negative), colony #2 (middle panel) is "partially" reprogrammed (mixture of CD44 and TRA-1-60 positive cells), and colony # 3 (right panel) appears to be nearly "fully" reprogrammed (most of the cells are TRA-1-60 positive).

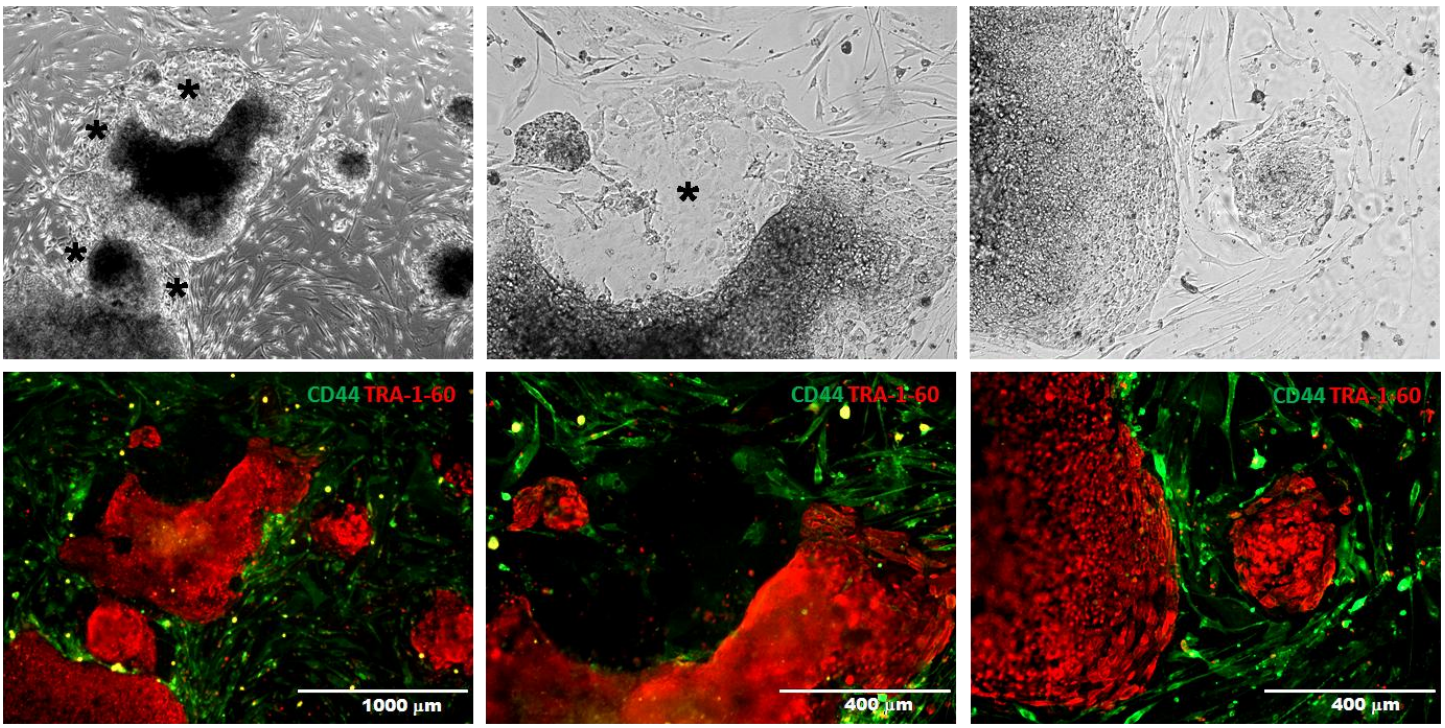

Figure 4 – At day 29 after initiating reprogramming, a culture growing on a MEF feeder layer was co-stained with live-cell qualified antibodies for CD44 (green, negative pluripotency marker that also stains the feeder cells) and TRA-1-60 (red, positive pluripotency marker). Phase and fluorescence images were taken at lower magnification (left panel) or at higher magnification (middle and right panels). The immunofluorescence staining identified colonies with a significant amount of differentiation (areas of cells lacking both CD44 and TRA-1-60 staining, indicated by asterisks in the left and middle panels) as well as "fully" reprogrammed / pluripotent colonies (TRA-1-60 positive) suitable for picking for further expansion and characterization (right panel).

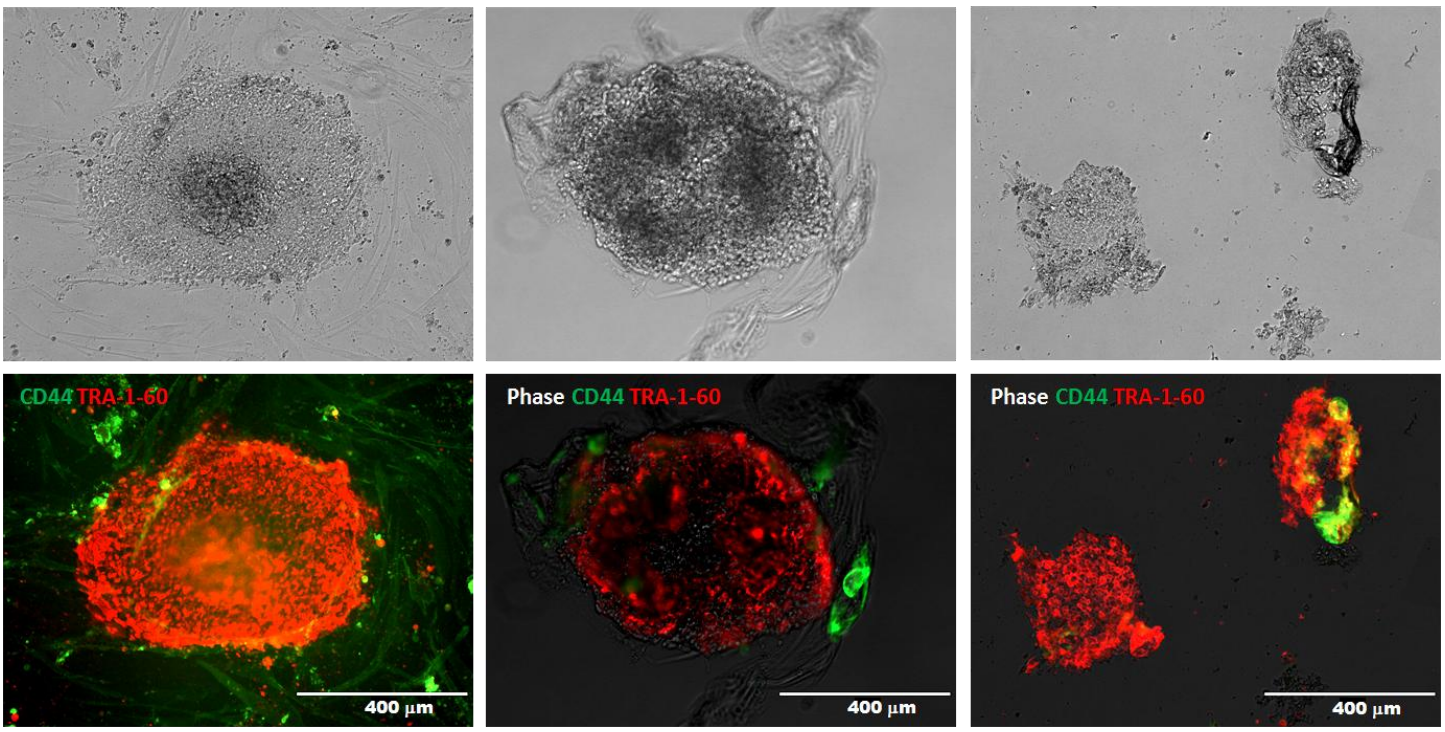

Figure 5 – At day 32 after initiating reprogramming, a culture growing on a MEF feeder layer was co-stained with live-cell qualified antibodies for CD44 (green, negative pluripotency marker that also stains the feeder cells) and TRA-1-60 (red, positive pluripotency marker). The immunofluorescence staining identified a "fully" reprogrammed / pluripotent colony (left panel, TRA-1-60 positive) that was picked and transferred to a new well (middle panel). After picking and transferring a colony to a new well, it was broken into smaller fragments (right panel) – successful transfer and fragmentation of a pluripotent colony was confirmed by immunofluorescence inspection of the transfer well.

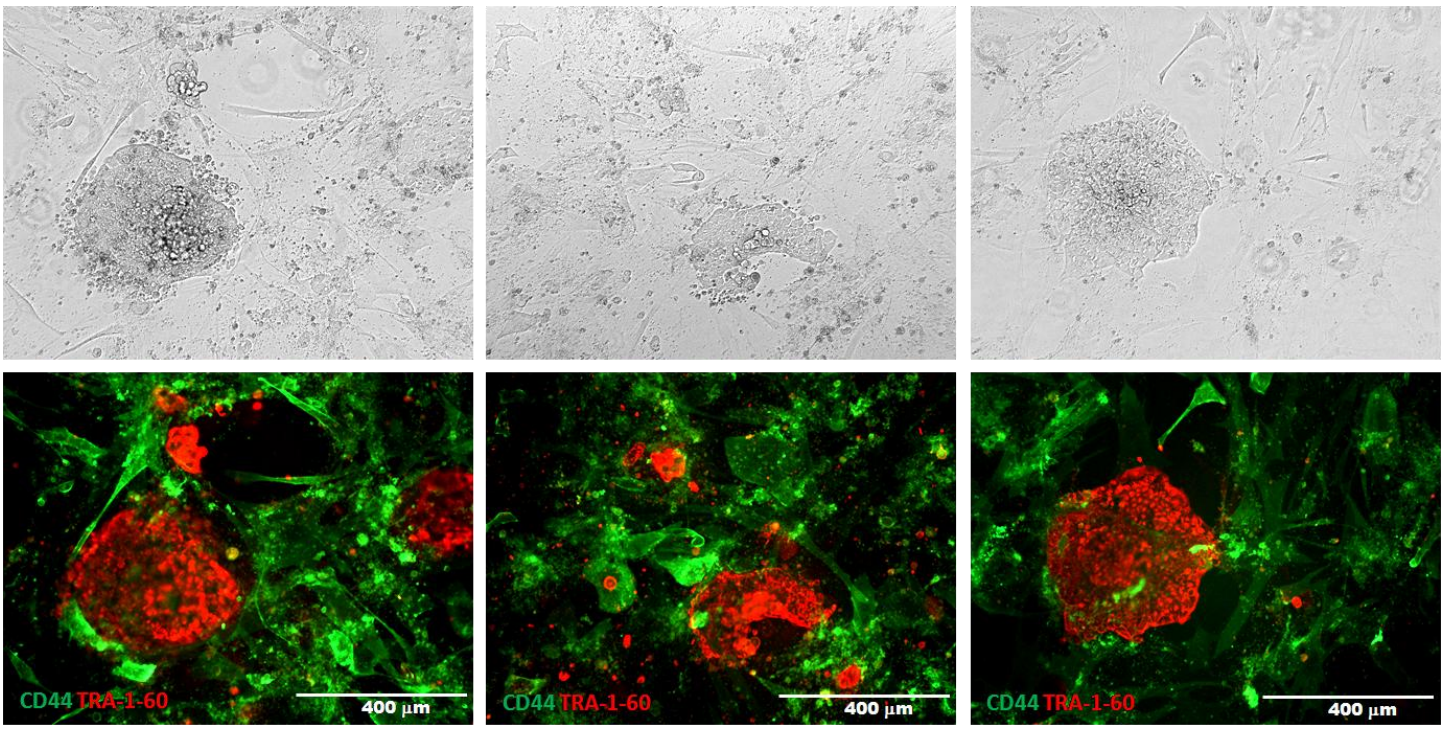

Figure 6 – Shown here is an additional staining example from reprogramming donor fibroblasts under feeder-free conditions. At 21 days after initiating reprogramming, a culture growing on vitronectin was co-stained with live-cell qualified antibodies for CD44 (green, negative pluripotency marker) and TRA-1-60 (red, positive pluripotency marker). Phase and fluorescence images from three fields of view are shown. Immunofluorescence staining confirms the presence of several pluripotent / "fully" reprogrammed TRA-1-60 positive colonies surrounded by non-reprogrammed CD44 positive cells.

Technical Support

Visit www.Lifetechnologies.com/support or email probestech@invitrogen.com or phone (800) 438-2209 or (541) 335-0353.