Novel Alkaline Phosphatase Live Stain to identify and isolate emerging iPSCs

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INTRODUCTION

We have previously reported the successful use of the highly efficient CytoTune™ iPS Sendai reprogramming particles[1] to generate iPSCs from human BJ fibroblasts. This footprint-free, non-integrating Sendai Virus mediated reprogramming, when coupled with the novel Alkaline Phosphatase Live Stain has generated iPSCs that are stable and fully reprogrammed[2]. AP Live Stain is a cell permeable substrate for the Alkalkine Phosphatase enzyme, which when kinetically turned over, expresses a fluorescent marker which then permeates out of the cell leaving no footprint behind. Unlike other AP stains, it is not a terminal dye, does not accumulate in the cell, is free from bioburden, mycoplasma free, low endotoxins and is nontoxic to the fragile iPSCs.

Here we report the sole use of AP Live Stain in the selection of emerging CytoTune™ iPS Sendai reprogrammed iPSCs derived on feeders and feeder free conditions. iPSC clones were tested for pluripotency via known cellular methods of characterization. All clones were expanded and banked. Selected clones were further analyzed for differentiation potential via undirected *in-vitro* differentiation, and were karyotyped.

MATERIALS AND METHODS

All reagents purchased from Life Technologies, unless otherwise noted.

Cell Culture: Feeder-dependent human H9 ESC and human iPSC were cultured in hESC media comprising of DMEM/F-12 (Cat #10565-018), 20% KSR (Cat #10828-028) and 4 ng/ml b-FGF (Cat # PHG0261) on Gibco® Mouse Embryonic fibroblast, irradiated (Cat# S1520100). Feeder-free cultures were maintained on StemPro® hESC SFM (Cat #A1000701), supplemented with 8 ng/ml b-FGF (Cat # PHG0261) on Geltrex® (Cat # A1413301) coated tissue culture dishes. hESC and hiPSC were routinely passaged using Collagenase , type IV (Cat #17104019).

Reprogramming: BJ human fibroblasts (ATCC®, CRL-2522™) were transduced with CytoTune™ iPS Sendai Reprogramming Kits (Cat #A1378001). A week after transduction, cells were seeded onto feeders in hESC media or onto Geltrex® (1:100) coated TC plates with StemPro® hESC SFM. 3 weeks post transduction, emerging iPSC colonies were identified and selected for, based on robust alkaline phosphatase activity.

Alkaline Phosphatase Live Staining: AP Live Stain (500X) (Cat #A14353) was diluted 1:500 (1X) in DMEM/F-12 media and directly applied onto adherent cell cultures. All staining procedures were conducted as per product specifications. Following visualization and or manual selection normal growth media was replaced and the PSCs were returned to normal culture conditions.

Pluripotent surface antibody staining: Antibodies for SSEA4, conjugated to Alexa Fluor® 594 (Cat #SSEA421) was used at a 1:500 dilution, and TRA-1-60 (Cat #411000) at 1:100 dilution, with appropriate Alexa Fluor® 594 secondary (Cat #A11005) were diluted in pre-warmed DMEM/F-12 media and directly applied on to adherent cell culture. All incubations and washes were performed at 37°C with pre-warmed DMEM/F-12 in order to maintain proper cell viability and normal live cell morphology.

Immuno-cytochemistry:. PSC cultures were fixed with 4% PFA, blocked and permeabilized prior to ICC. Pluripotent antibody staining was carried out on fixed cells using anti-Oct4 antibody (Cat # A13998) at 1:500 dilution and Alexa Fluor® 594 secondary (Cat #A11037) and anti-Alkaline Phosphatase antibody (BD Pharminogen Cat # 561432) and Alexa Fluor® 488 secondary (Cat #A11001). Differentiation primary antibodies used were anti-AFP (Cat #180003) at 1:1,000, anti-β-III-tubulin (Cat # 480011) at 1:1,000, and anti-SMA (cat# 180106) at 1:100, and probed with Alexa Fluor® 594 secondary (Cat #A11005).

Cell Viability Assay: PSCs were grown in feeder-free conditions, tested with appropriate conditions and viabilities were measured using the PrestoBlue® Cell Viability Reagent (Cat# A13261), 18 hours after treatment.

RESULTS.

Figure 1. Schematic of Staining Workflow

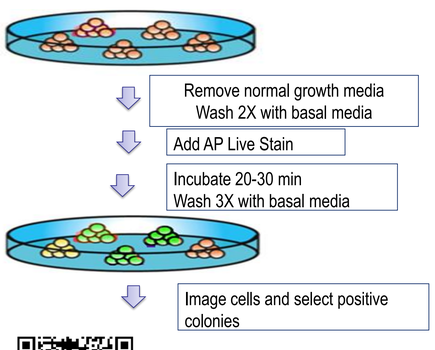
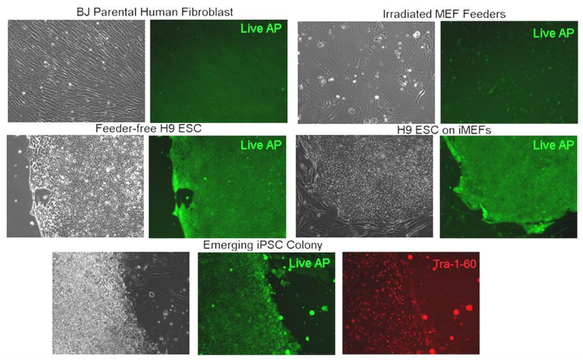
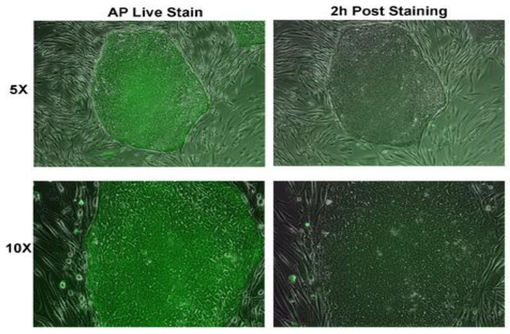


Figure 2. AP Live Stain can be used to identify pluripotent stem cells via a green fluorescent signal



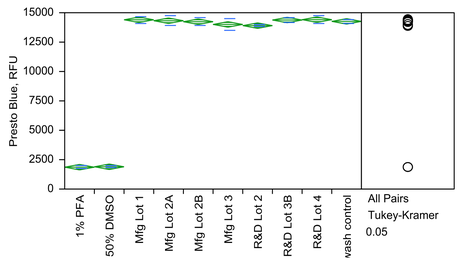
Alkaline Phosphatase activity has been shown to be highly up-regulated in pluripotent stem cells. Here we show the differential expression of AP activity, as gauged by the AP Live Stain in mouse feeder cells, human fibroblasts and H9 ESC (grown in feeder dependent and independent conditions). AP live stain correlates with early pluripotent markers, such as TRA-1-60, in emerging iPSC.

Figure 3. AP Live Stain is used a s a transient method of detecting Alkaline Phosphatase activity



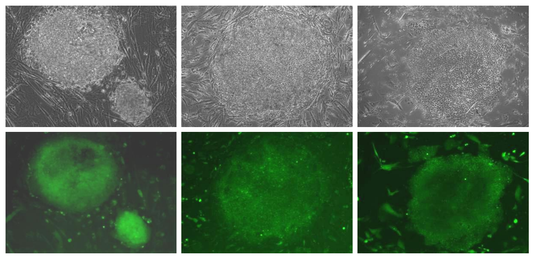
AP Live stain was applied to H9 ESC growing on iMEFs. Following the removal of staining solution, the fluorescent signal disappears from the stained cells within 2 hours initial treatment, without any residual footprint.

Figure 4. Use of AP Live Stain does not affect cell viability



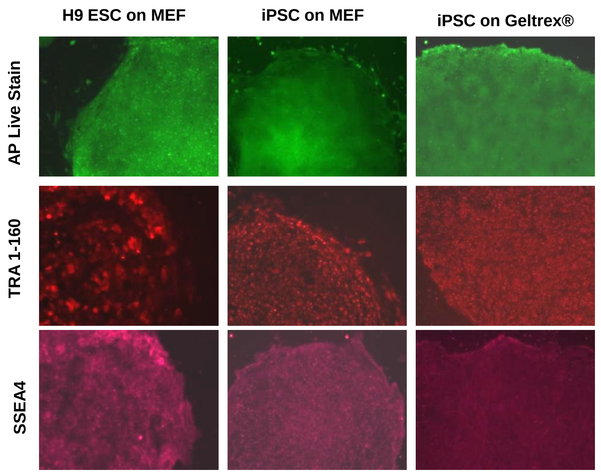
AP Live Stain does not diminish cell viability after its use. Here we demonstrate normal growth patterns for H9 ESC treated with AP Live Stain and allowed to recover for 20 hours. All trials demonstrated normal growth compared to un stained (wash) controls and do not show decrease viability, as compared to known cell disruptors, 1% PFA and 50% DMSO, as measured by PrestoBlue® Cell Viability Reagent.

Figure 5: AP Live Stain can be successfully used in iPSC selection during reprogramming



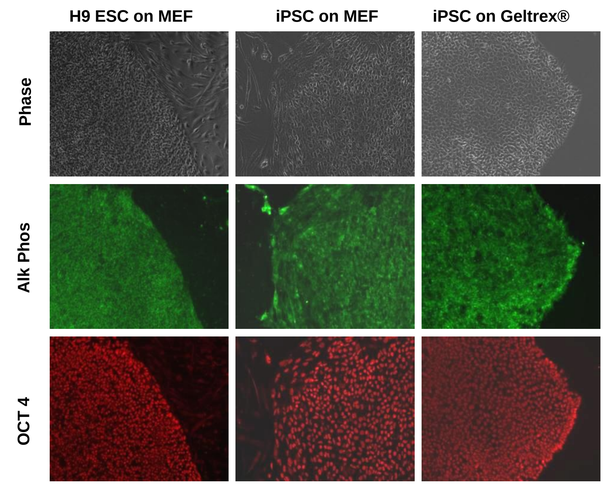
Alkaline Phosphatase Live Stain was used on CytoTune™ reprogrammed fibroblasts, seeded on both feeders and feeder-independent cultures to identify emerging pluripotent colonies.

Figure 6. Non-invasive characterization of pluripotency



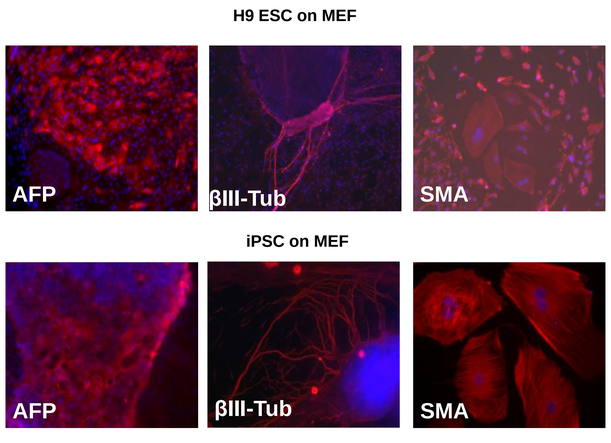
Established iPSC clones continue to expand normally and continue to express pluripotent markers. Non-invasive surface antibody staining with TRA 1-60 and SSEA4 and the re-confirmation of alkaline phosphatase activity through AP Live Staining demonstrates uniform pluripotency, as compared to H9 ESC.

Figure 7. Characterization of pluripotency via immuno-cytochemistry



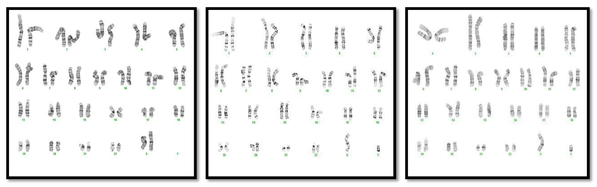
Emerging iPSC clones, selected using AP Live Stain were expanded in feeder dependant and feeder independent cultures. Colonies were fixed and probed with antibodies for OCT4 and Alkaline Phosphatase to confirm pluripotency, as compared to established H9 ESC.

Figure 8: Pluripotency potential via *in-vitro* undirected differentiation



Pluripotency was confirmed via the differentiation potential through *in-vitro* undirected differentiation of established iPSC (bottom panel) and H9 ESC (top panel). PSCs differentiated to cells representative of the 3 embryonic germ layers when probed with antibodies for: alpha-fetoprotein (AFP) for endoderm, beta-III-Tubulin (β-III-Tub) for ectoderm, and smooth muscle actin (SMA)for mesoderm.

Figure 9. Cytogenic Analysis to determine karyotype



H9 on MEF
46, XX [20]

iPSC on MEF
46, XY [20]

iPSC on Geltrex®
46, XY [20]

Cytogenic analysis of G-banded metaphase cells of CytoTune™ derived iPSC demonstrated normal karyotypes.

CONCLUSIONS

❖Alkaline Phosphatase Live Stain allows for robust fluorescence detection and further proliferation of stained pluripotent stem cells. It may be used not only during initial selection on the master reprogramming plate for the iPSC generation workflow but also can be used multiple times throughout expansion as a confirmation of continued pluripotency without effecting cell viability or culture conditions.

❖Cellular morphologies of emerging iPSC picked with AP Live Stain showed compact colony formation with defined edges on feeders with morphology characteristic of embryonic stem cells.

❖ CytoTune™ iPS Sendai reprogramming in fibroblasts continues to yield high quality iPSC when selected for with AP Live Stain in both feeder-dependant and feeder-free conditions.

❖AP Live Stained picked colonies, derived on feeder free conditions demonstrated similar growth and morphologies as ESCs that were adapted to similar culture conditions.

❖ iPSC derived on feeders and feeder free conditions demonstrated similar expression of pluripotent surface markers as judged by live cultures probed with antibodies against know pluripotent markers SSEA4 and TRA1-60. Results were similar to what has been established in human ESC.

❖iPSC were expanded to over 20 passages and shown to continue to express intercellular pluripotent markers OCT4 and Alkaline Phosphatase as measured by traditional immun-cytochemistry.

❖CytoTune™ iPS Sendai reprogramming generated iPSC that maintained a normal karyotype and can spontaneous differentiate into liver cells, neural cells and smooth muscle cell types, representative of the three germ layers, as a measure of pluripotency as compared to traditional ESC models.

REFERENCES

1. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. (2009) Proc Jpn Acad Ser V Phys Biol Sci. 85(8): 348-362.
2. Singh U, Quintanilla RH, Grecian S, Gee KR, Rao MS, Lakshmipathy U. (2012) Stem Cell reviews and reports. [Epub Mar 2012].

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