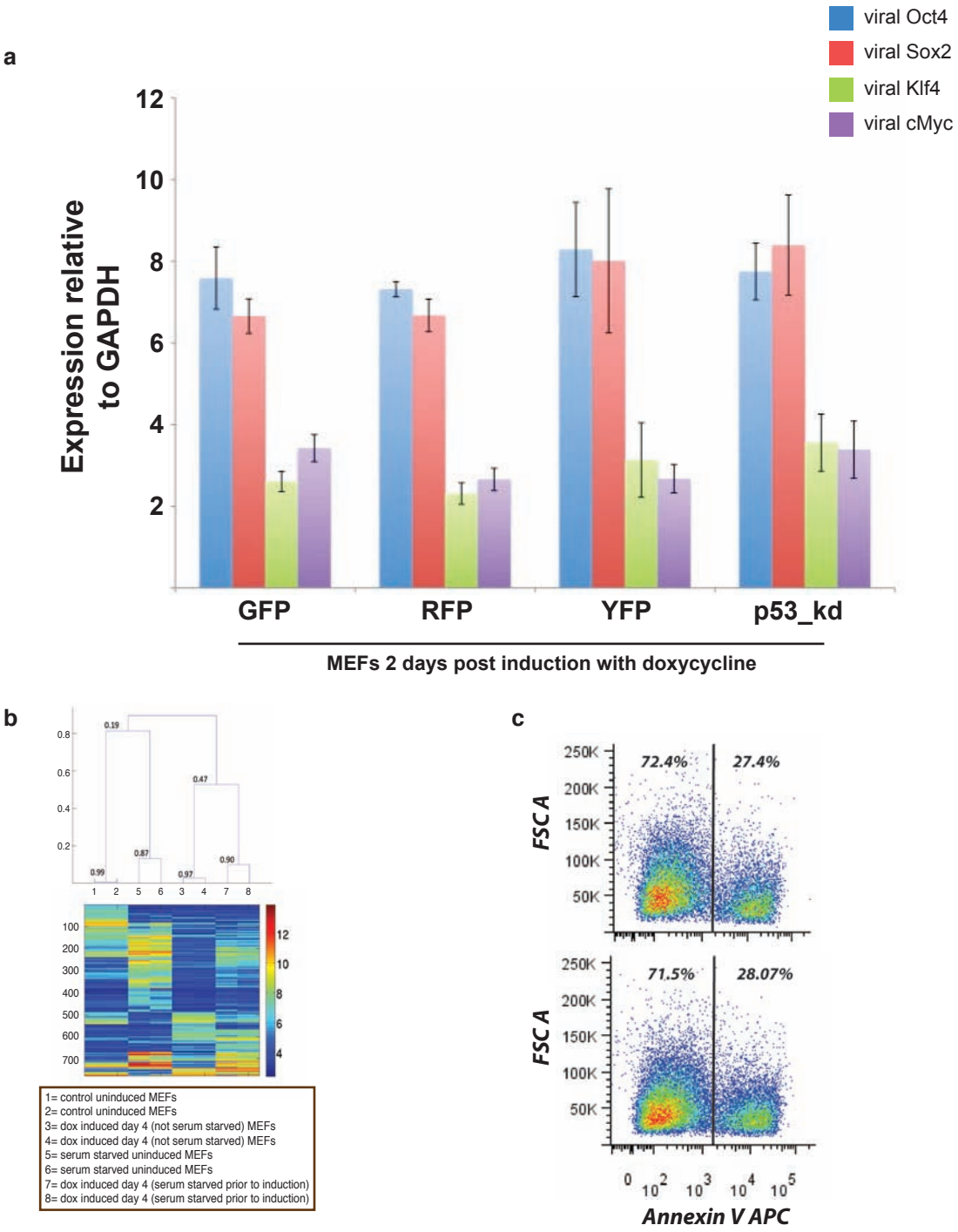
Characterization of an inducible Mouse Embryonic Fibroblast (MEF) imaging system for studying direct reprogramming



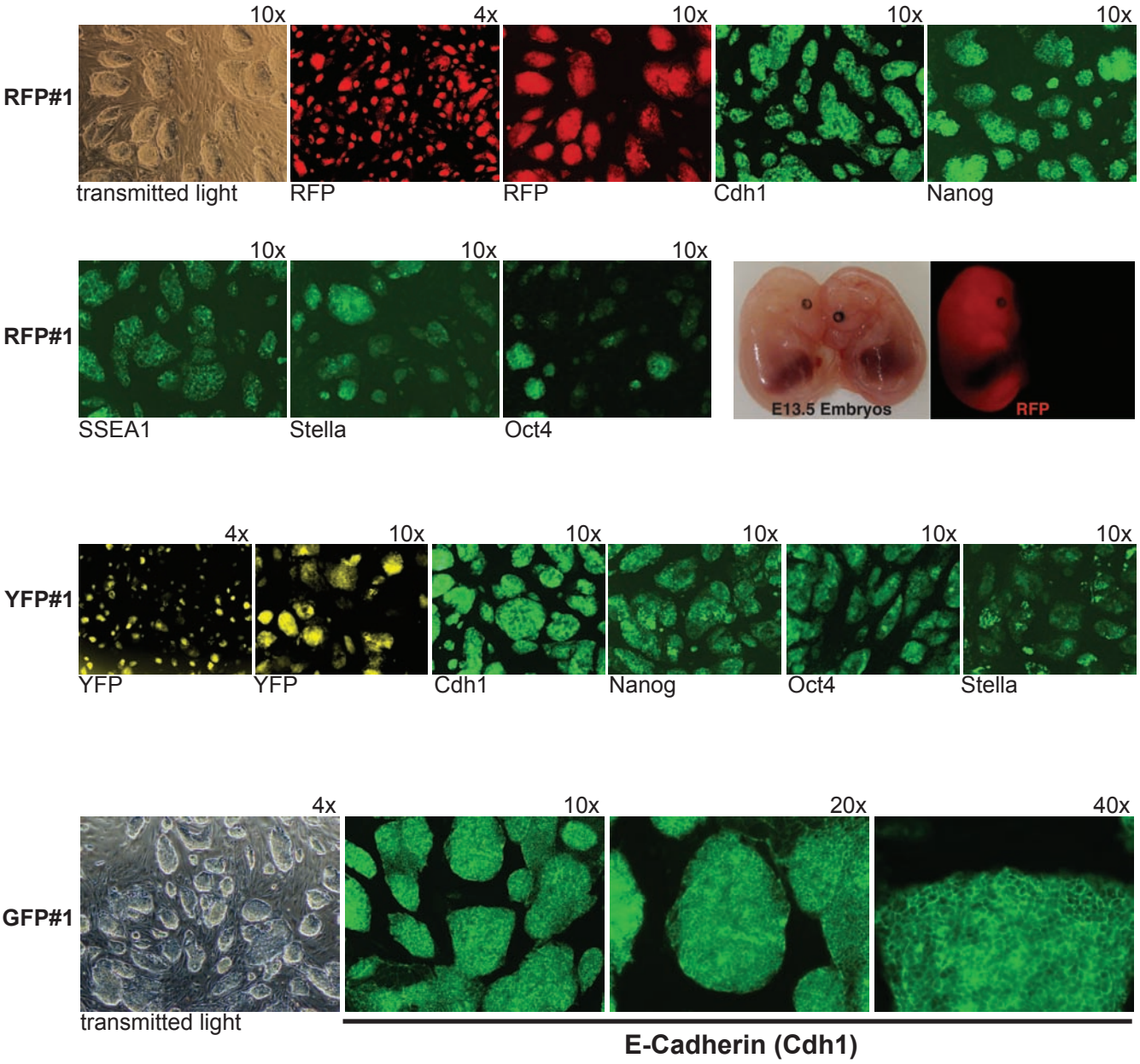
a) Expression of viral transgenes was measured by Real-Time qPCR relative to GAPDH two days after adding doxycycline against uninduced fractions that are cultured in parallel. Each bar represents the mean expression data for triplicate fluorescently labeled populations.

b) Dox induction reduces expression differences between serum starved and non-starvedcells. Shown is hierarchical clustering of expression profiles from 4 conditions, each in duplicate: MEFs, Dox-induced MEFs, serum-starved MEFs, Dox-induced MEFs that were previously serum-starved. A subset of 779 genes that showed at least a 2-fold induction within the data set are used or representation. Induction by Dox results in much higher correlation (0.47 vs. 0.19 over this gene set) between serum starved and non-starved populations.

c) Apoptotic response to reprogramming factors within clonally induced MEFs was confirmed by flow cytometry against extracellular Annexin V using an APC conjugated antibody (BD Pharmingen) and is similar across induced populations.

Smith *et al.* Supplementary Figure 1

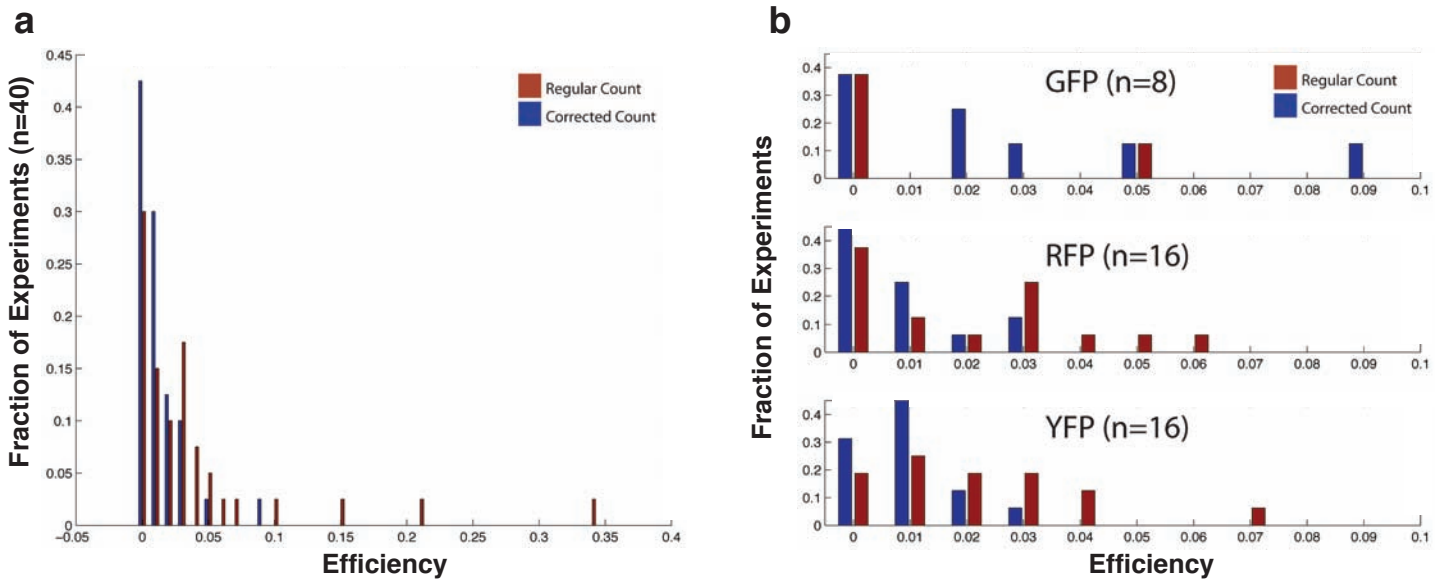
Characterization of iPS cell lines.



From several of our experiments we isolated stable, Dox-independent iPS cell lines. Here we show characterization of one representative line per color (RFP, YFP and GFP) using Cdh1, Nanog, SSEA1, Stella, and Oct4 immunostaining, which co-occur in iPS cells generated using the dox-inducible system with high fidelity. We also provide evidence of functional pluripotency by generating a high contribution chimera as seen next to a control embryo.

Smith *et al.* Supplementary Figure 2

Distribution of reprogramming efficiencies is similar across experiments

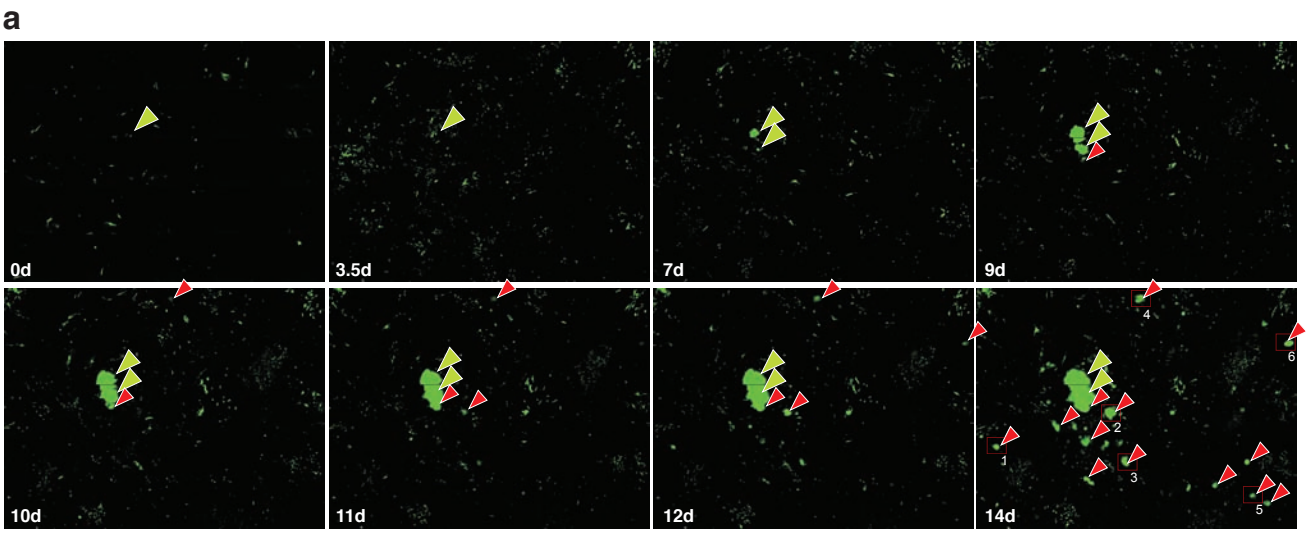


a. The efficiencies of 40 fluorescently labeled populations before and after normalization against acquired satellite colonies. Before correction, the relative range of iPS cell colonies is broader and extends into atypically large efficiencies per founding population. The distribution after correction is more defined and establishes the absolute efficiencies by which a single cell progresses to form iPS cell colonies.

b. Representative efficiencies per wavelength exhibit a similar distribution before and after normalization as in a.

Smith *et al.* Supplementary Figure 3

Satellite colonies



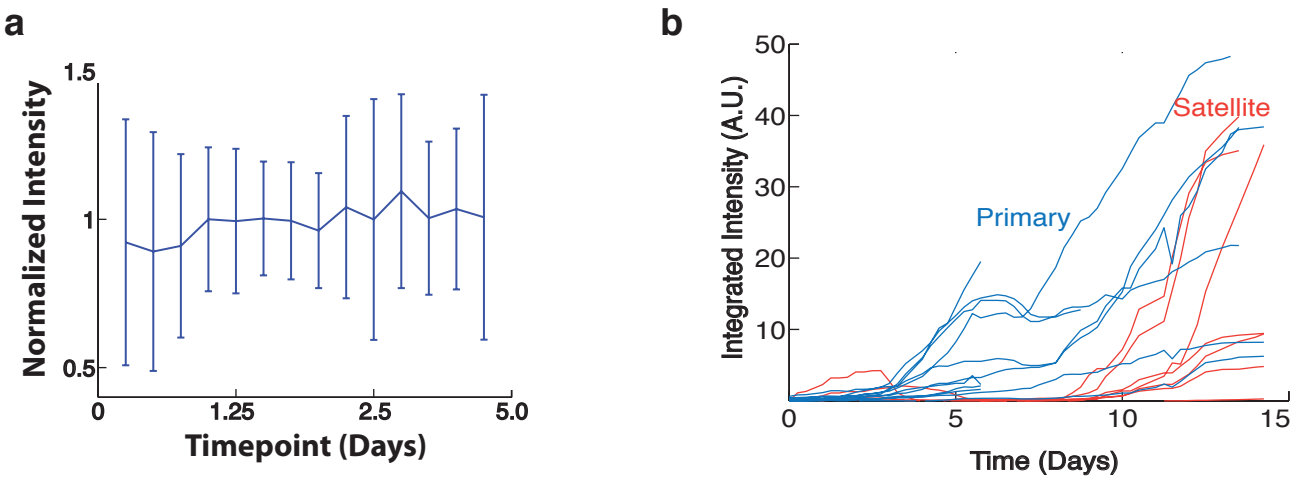
a. GFP labeled satellite colonies without unique origins over a global 5x5 field in 10x magnification. Satellite colonies (a subset highlighted with red arrowheads) typically become macroscopically visible after day 6-8 and the formation of primary colonies (yellow arrowheads) without a traceable origin (see Supplementary Movie 2).

b. A subset of the images shown in panel a) that was also used in Figure 2a and b. Shown are additional higher magnification images of all 6 colonies. All clearly demonstrate that the colonies arose at later time-points without a source cell in the area.

c. We induced MEFs and sorted single cells into wells of several 96 well plates four days after induction. Shown are 8 examples of a single MEF giving rise to a primary and satellite colonies. The two images on the right (#8) are from two distant corners of the same well. Images are 4x and were scored on day 14.

Smith *et al.* Supplementary Figure 4

Scoring of primary or secondary iPS cell origin is confirmed by digital image analysis

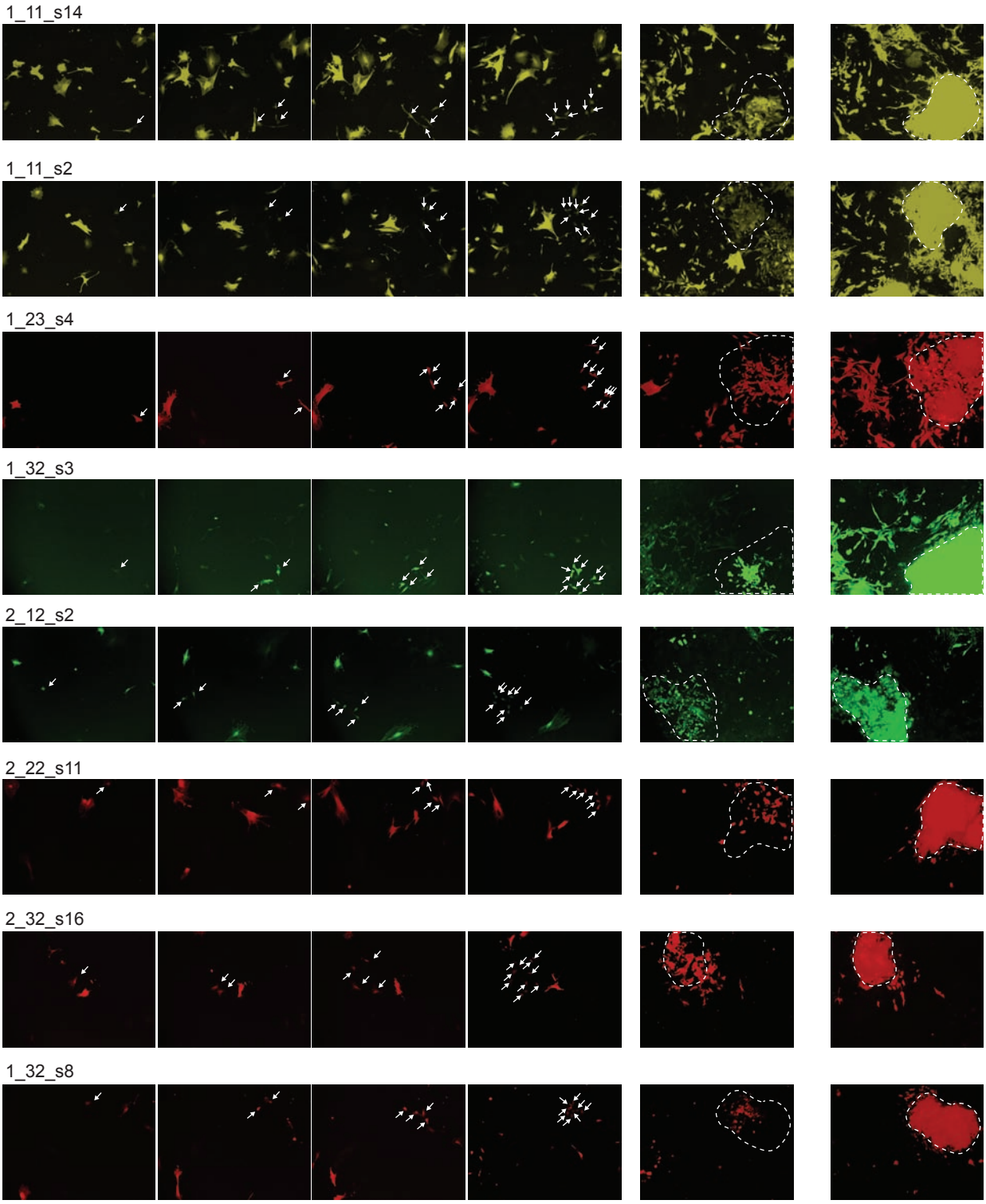


a. Discrete fluorescently labeled lineages display a minimal dynamic range over multiple days when normalized to the mean intensity of the original cell at t=0 days. Representation is the standard deviation of the median fluctuation of the fluorescent intensity for all lineages analyzed over the first 4 days, when single cells can be most easily discerned.

b. Given the constitutive nature of our fluorescent proteins, a bounding rectangle can be determined for each of the analyzed satellite (red) and primary (blue) iPS cell lineages. Total fluorescent intensity in the rectangle was summed for each time point. This highlights the continuous and early appearance of primary colonies compared to the much later and abrupt appearance of satellites.

Smith *et al.* Supplementary Figure 5

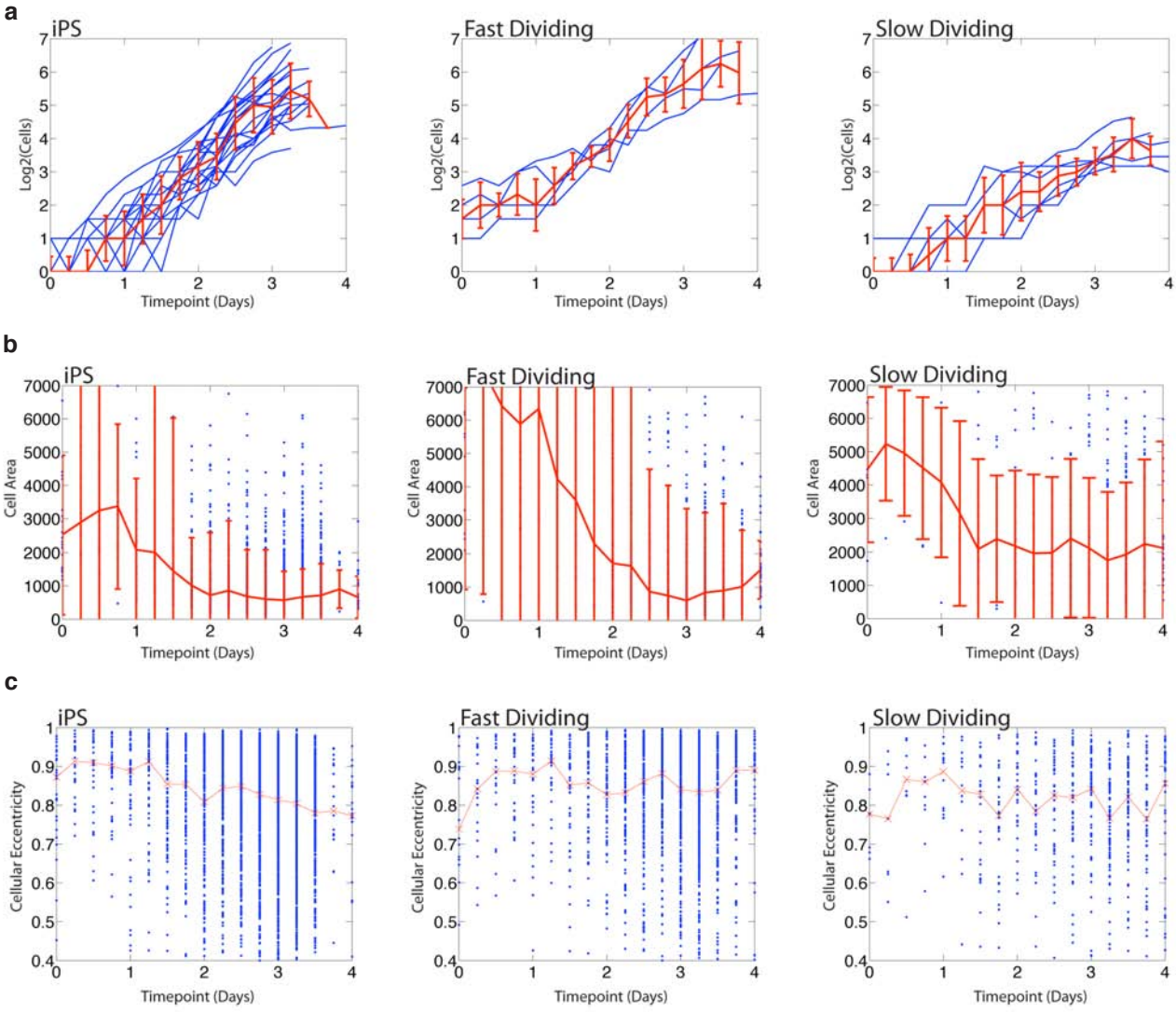
Back-traced iPS cell lineages

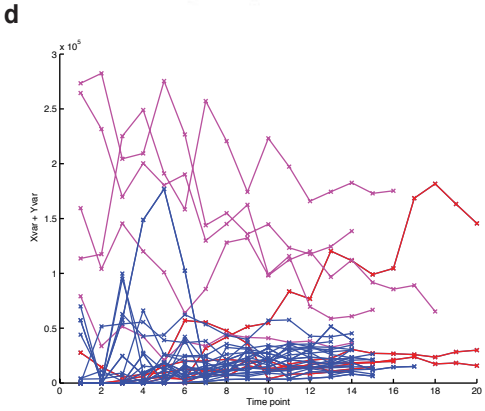


Shown are eight additional lineages. The first four images show the first 3-5 divisions, the next image from left to right shows compaction and the last image of the final colony. Arrows highlight the cells within the reprogramming lineages.

Smith *et al.* Supplementary Figure 6

Growth rate, cellular area and morphology distinguish the early response of reprogramming cells





a. Log2 growth curves of iPS cell forming (n=19), Fast (n=5), and Slow (n=5) Dividing lineages over time. The Log2 linearity of cell number evident in iPS cell forming cells demonstrate a proliferative rate of 12.2±2.8 hrs that is initiated upon the first cell division, indicative of a symmetrically conferred growth potential specific to these cells.

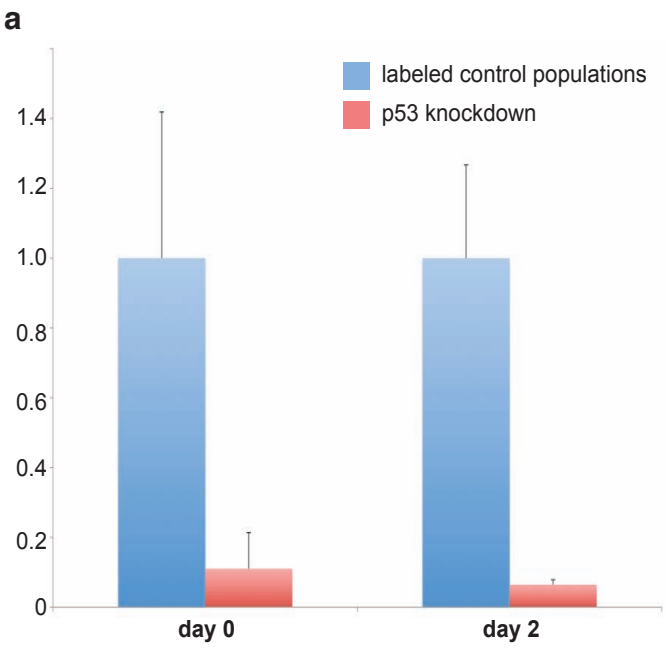
b. Cellular area of different responding lineages over time. iPS cell forming lineages demonstrate a rapid progression to an iPS-like size within the first 2-3 days that is present in the majority of cells as they proliferate. Comparatively, fast dividing (FD) fibroblast-like populations also exhibit a change in cell size, presumably an attribute of oncogene-mediated transformation, but this phenotype occurs less frequently and is not stable. The range of cell sizes is more inconsistent and generally increases by day 4 when cells reassume a more elongated, mesenchymal morphology. The relatively small size range present in iPS cell forming lineages is an indicator of the symmetrical inheritance of this trait, as precursor cells give rise to successively smaller progeny until a stable state is reached.

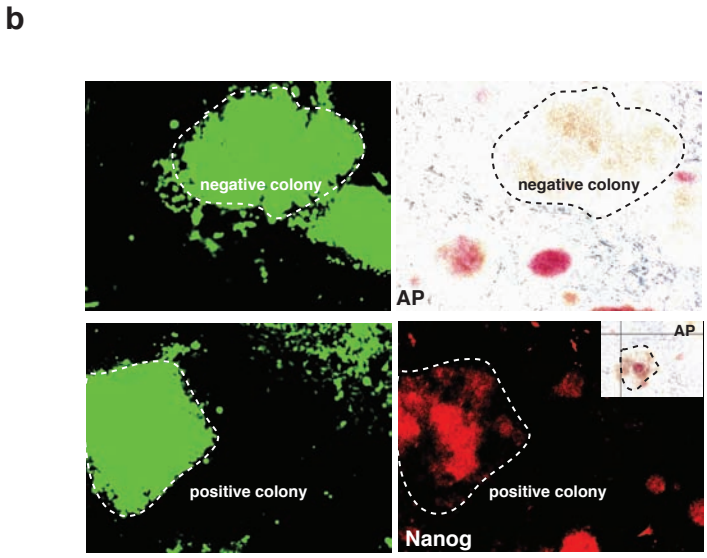
c. Cellular eccentricity as a metric for cell shape in characterized responding morphologies. Over time, iPS cell forming lineages demonstrate a steady decrease in eccentricity that is not observed in fast or slow dividing fibroblast populations.

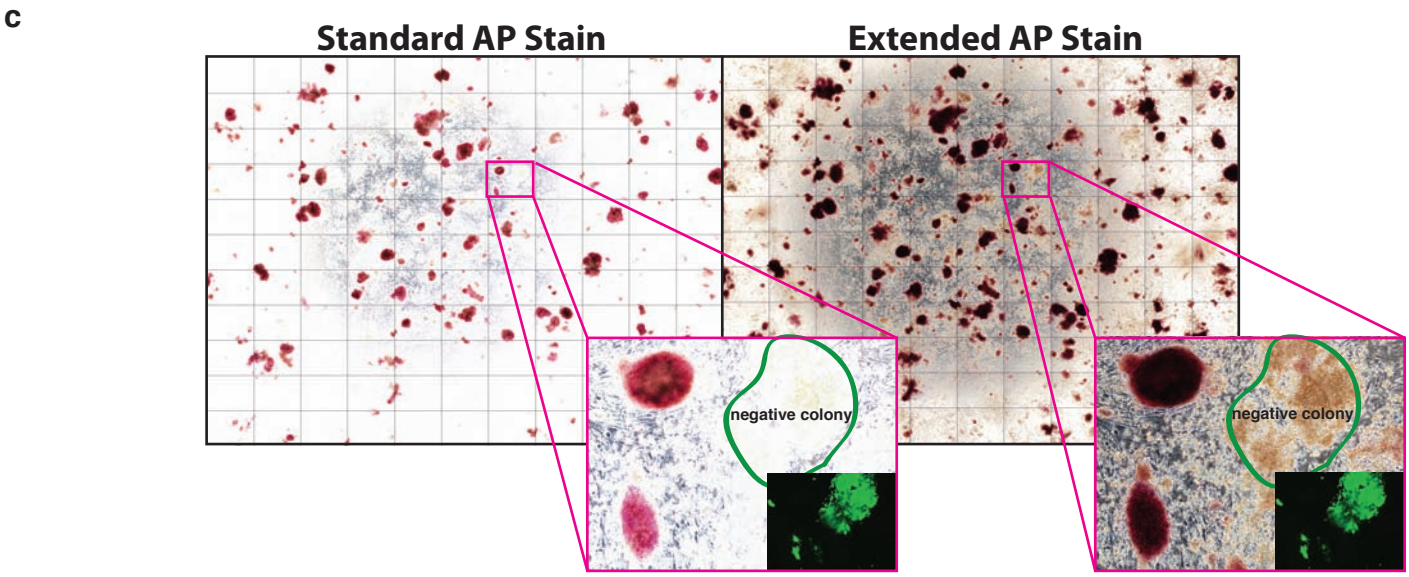
d. Lineage spread across time for the iPS (blue), FD (purple) and SD (red) lineages. The spread is measured as the sum of spatial variances along the X and Y directions and validates observations that iPS cell forming lineages grow more uniformly as tight clusters than more mesenchymal, fast dividing lineages.

Smith *et al.* Supplementary Figure 7

Characterization of p53 targeted inducible fibroblasts and distinct colony morphologies







a. Shown is the relative depletion of p53 mRNA in the p53 shRNA transduced MEFs compared to the labeled control populations on day 0 and day 2 post induction (n=3 independent biological replicates).

b. Morphological characteristics of pluripotency positive and negative colonies that arise in p53-depleted cells. Upper panel shows a representative pluripotency marker negative colony, which has basal level signal for AP but is distinguishable from pluripotency positive colonies (lower panels). The emergence of ES-factor negative, colony-resembling foci that are distinct from the fast dividing fibroblast (FD) or true iPS colonies is unique to the p53 depleted cells and has not been otherwise observed for control secondary MEFs.

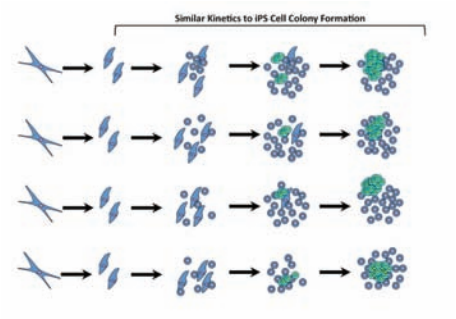
c. With extended AP stain, the global field exhibits a heightened basal signal from these pluripotency negative colonies that may suggest the capacity of p53 depletion to stabilize partially reprogrammed, terminal intermediates. Control iPS cell colonies are present in the field and serve as a positive control for AP signal.

Smith *et al.* Supplementary Figure 8

Models for iPS cell lineage reprogramming dynamics using live imaging data

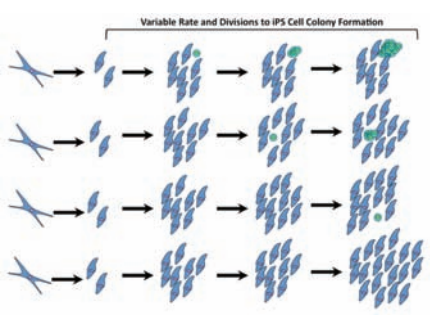
a

Deterministic Multi-Step Model

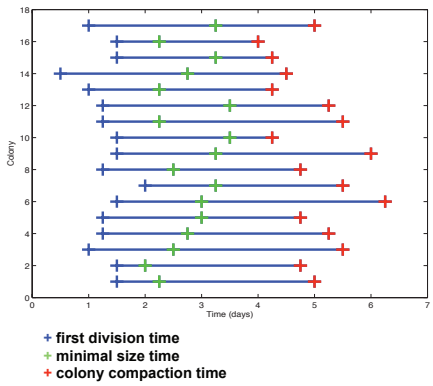


b

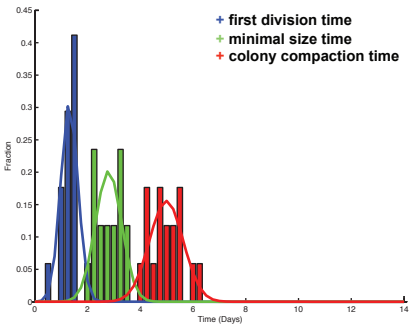
Stochastic One-Step Model



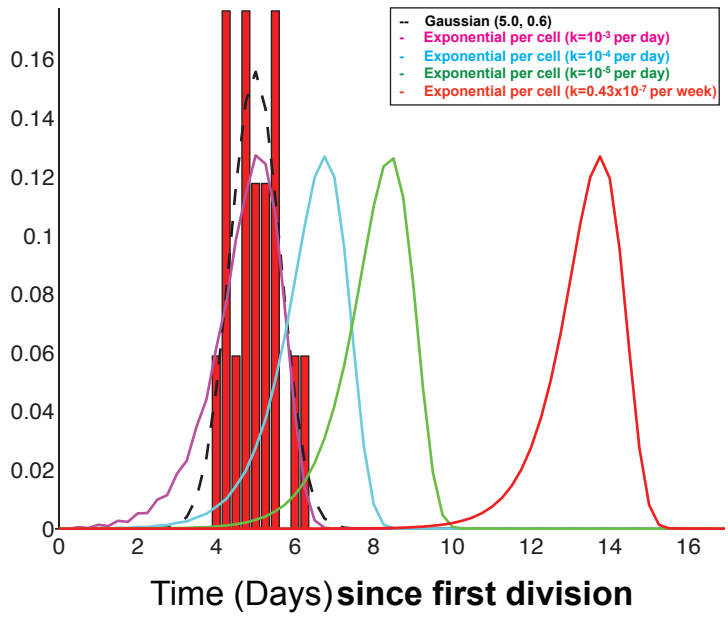
c



d



e



a. A schematic representation of reprogramming progression under two distinct models. In a deterministic multi-step model (left) all the lineages advance through multiple steps (e.g. size reduction, clustering) toward reprogramming.

b. In a stochastic one-step model 10 (right) every cell in every lineage has an equal chance of reprogramming in a single step.

c. Timing of landmark events in each of the analyzed iPS cell colonies. The events scored are time of first division (blue cross), time of reaching minimal median size (green cross) and time of colony compaction (red cross).

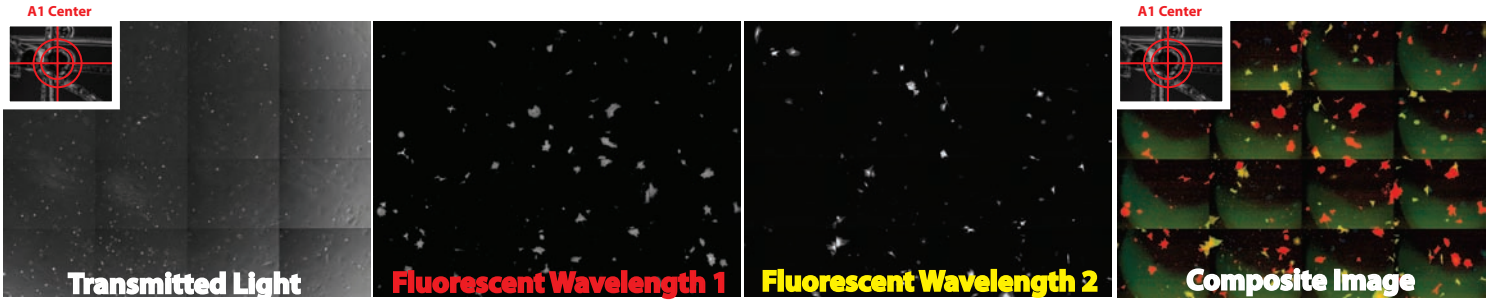
d. Event timing histograms for first division time (blue), time of minimal size (green) and colony compaction time (red) for the analyzed iPS colonies. Solid lines show fit to Gaussian distributions (first division: mean 1.3d, std 0.3d; minimal size: mean 2.8d, std 0.5d; colony compaction: mean 5.0d, std 0.6d)

e. Comparison of observed distribution of colony compaction times (red bars) to the best fit Gaussian distribution (black dashed lines) as well as one-step stochastic models with different k reprogramming rate parameters (purple, cyan, green and red curves). The k in red is as described for reprogramming blood lineages using a similar inducible system (*Ref. 10)*.

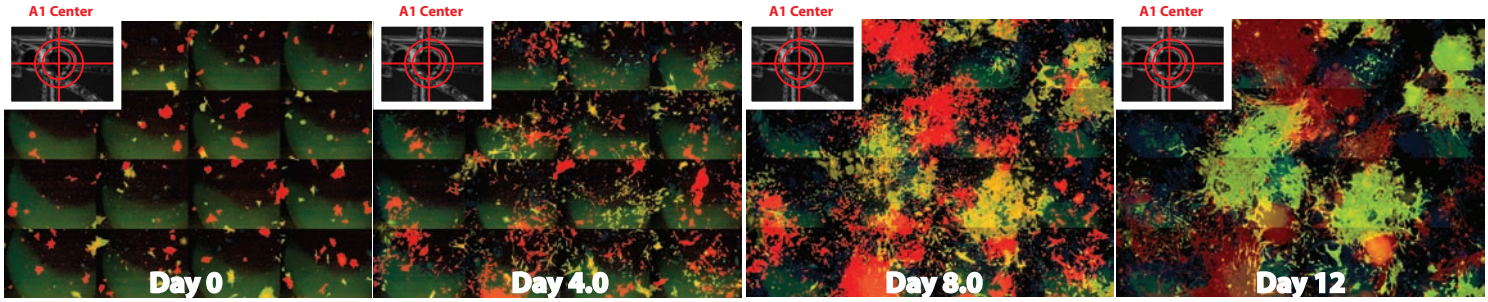
Smith *et al.* Supplementary Figure 9

Method for Tracking Reprogramming Populations

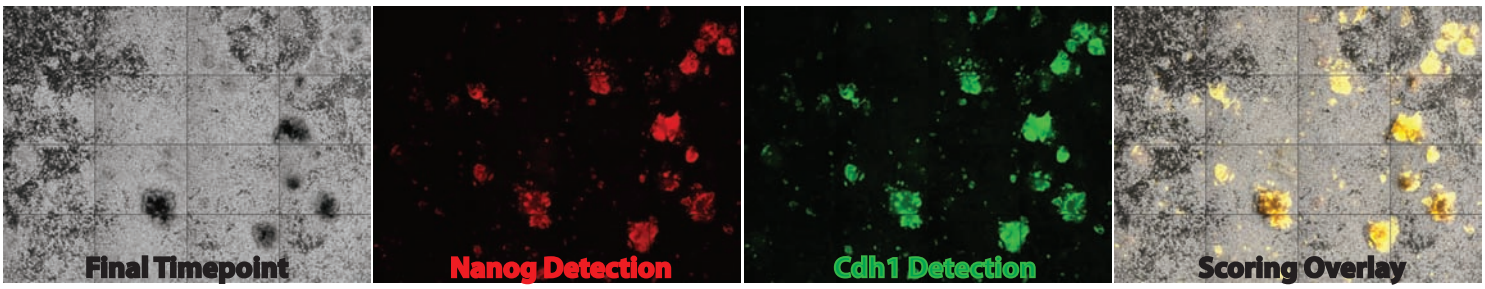
a. Single Timepoint: Multi-Image and Multi-Wavelength Acquisition



b. Multiple Timepoints: 14 Day Population Tracking



c. Terminal Characterization: Fixation and Immunostaining of Traced Lineages



a. Fluorescently labeled cells were imaged across multiple wells by manually defining an artificial “bulls eye” position (A1 center, superimposed) to reset a calibrated XY plane for each time point. Distinct fields in phase contrast and up to 4 fluorescently labeled populations were acquired per time point and stitched together as a complete field. Single wavelength acquisitions were then normalized and compiled as overlays to analyze global data.

b. Acquisitions proceeded over a 12 to 14 day imaging period with positional information retained and compiled into reprogramming timelines (Supplementary Movie 7).

c. At the terminal time point, reprogramming populations were fixed with 4% paraformaldehyde and stained for at least two endogenous pluripotency markers, Nanog and E-cadherin (Cdh1).

Smith *et al.* Supplementary Figure 10

Supplementary Movie Legends:

Supplementary Movie 1: Time-lapse movie of a single (1/16 of the 4x4) monitored site (upper right corner of **Fig. 1a,b,c** and same as **1d**). The movie follows the YFP labeled fibroblasts (#16 in **Fig. 1b**) as it divides and forms a colony over the course of 12 days. This MEF exhibits an immediate change in its proliferative behavior and size maintenance, rapidly forming many small cells that eventually give rise to multiple distinct iPS colonies within the formed cluster **(shown in Figure 1d)**. Non responding induced MEFs are apparent in the upper right and lower left hand corner for immediate comparison to the reprogramming lineage; these cells divide more slowly and maintain large, asymmetrical mesenchymal characteristics. Time in days is shown and the arrow in the first frame points to the source cell.

Supplementary Movie 2: Time-lapse movie of a global 5x5 field at 10x magnification in phase contrast and with GFP-labeled MEFs highlighted over a 14 day experiment. A single primary colony emerges at Day 4 while subsequent satellites continue to accumulate from Day 6 to the termination of the experiment at Day 14.

Supplementary Movie 3: Time-lapse movie following a YFP-labeled MEF progressing to a lineage in which multiple iPS cell colonies (~6) are formed (**Fig. 2d** shows selected images of this movie in grayscale). These subpopulations within the responding lineage can be clearly demarcated before unique iPS cell colonies are observed. Within the earliest time points, an additional YFP labeled responding population can be observed at the left, but these cells do not progress through the preliminary size reducing response, are not accountable for an iPS cell colony, and are lost or scored as non-proliferative by Day 6. Time in days is shown and the arrow in the first frame points to the source cell.

Supplementary Movies 4a-d: Distinct morphologies of responding MEFs characterized from time-lapse imaging.

a. **Arrested/Apoptotic (A)** responses in GFP, RFP, and YFP labeled MEFs. Several of these cells initially exhibit attributes of a positive response to factor

induction as outlined in text but either arrest/apoptose or transition to a slower proliferative rate.

b. **Slow dividing (SD)** YFP-labeled MEFs that do not reprogram, but slowly divide and maintain many of the morphological attributes of the original MEF population.
c. **Fast Dividing Fibroblast (FD)** YFP labeled MEFs that demonstrate a transition to a rapid cell cycle but become more striated and grow as a mesenchymal monolayer. Note, that within this fast dividing fibroblast population, three ectopic, unlabeled satellite colonies emerge within the final 4 days of the 14 day time series.
d. **iPS Forming (iPS)** A RFP labeled MEF exhibiting an immediate change in proliferative rate and cell size, forming small clusters of dividing cells that lead to the formation of compacted subpopulations which give rise to pluripotency marker positive iPS cells.

Supplementary Movie 5: Time-lapse movie of a MEF infected with an EGFP labeled p53 targeting shRNA (Upper Panels) compared to an internal RFP labeled control MEF (Lower Panels) within the same experiment (**Fig 4b** shows selected images from these movies). Size and proliferative rate are nearly identical within the first four days, but only the RFP labeled MEF progeny continue along an expected trajectory to a compact iPS colony whereas the p53 depleted MEF slows its proliferative rate and forms a colony that is less compact compared to the wild type control. Note that this p53 KD derived colony remains negative for classical pluripotency markers at the final time point (14 days). Time in days is shown and the arrow in the first frame points to the source cell.

Supplementary Movie 6: iPS cell colonies exhibit conserved formation dynamics. Shown are 12 independent iPS cell forming lineages imaged from the time of factor induction to termination after 14 days. As shown in **Figure 5,** each population is governed by near identical kinetics and concludes in a tightly distributed time of primary colony emergence that is unlikely to be dictated by a single low probability stochastic event.

Supplementary Movie 7: Representative population wide overlays of RFP and YFP labeled inducible MEFs over a 12 day experiment and from which the YFP labeled fibroblast labeled #16 in **Figure 1** was identified as a cell of origin for a primary iPS cell colony. Similar overlays were generated for every experiment and used to both identify primary colonies for downstream analysis and to gauge global reprogramming trends.