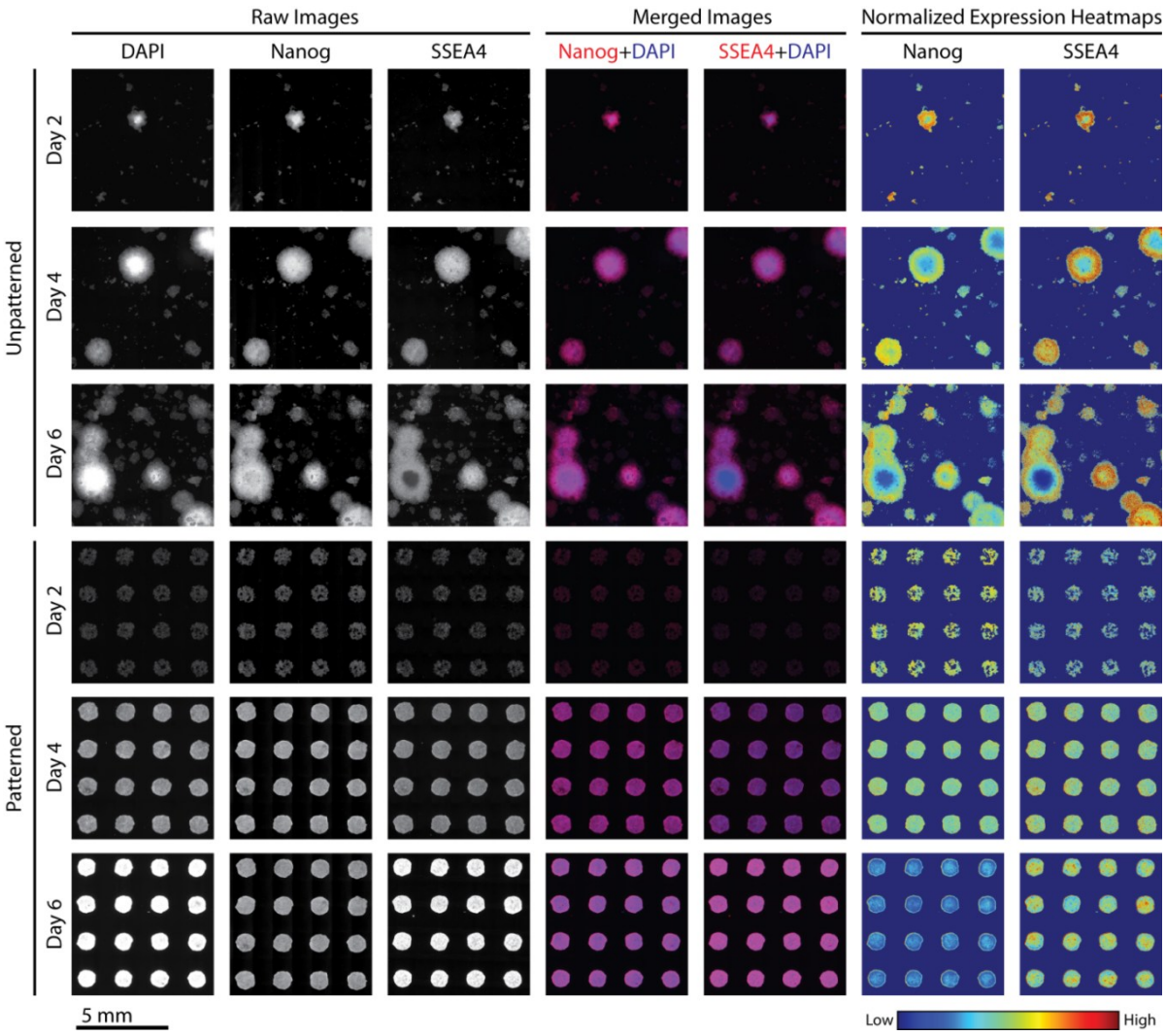
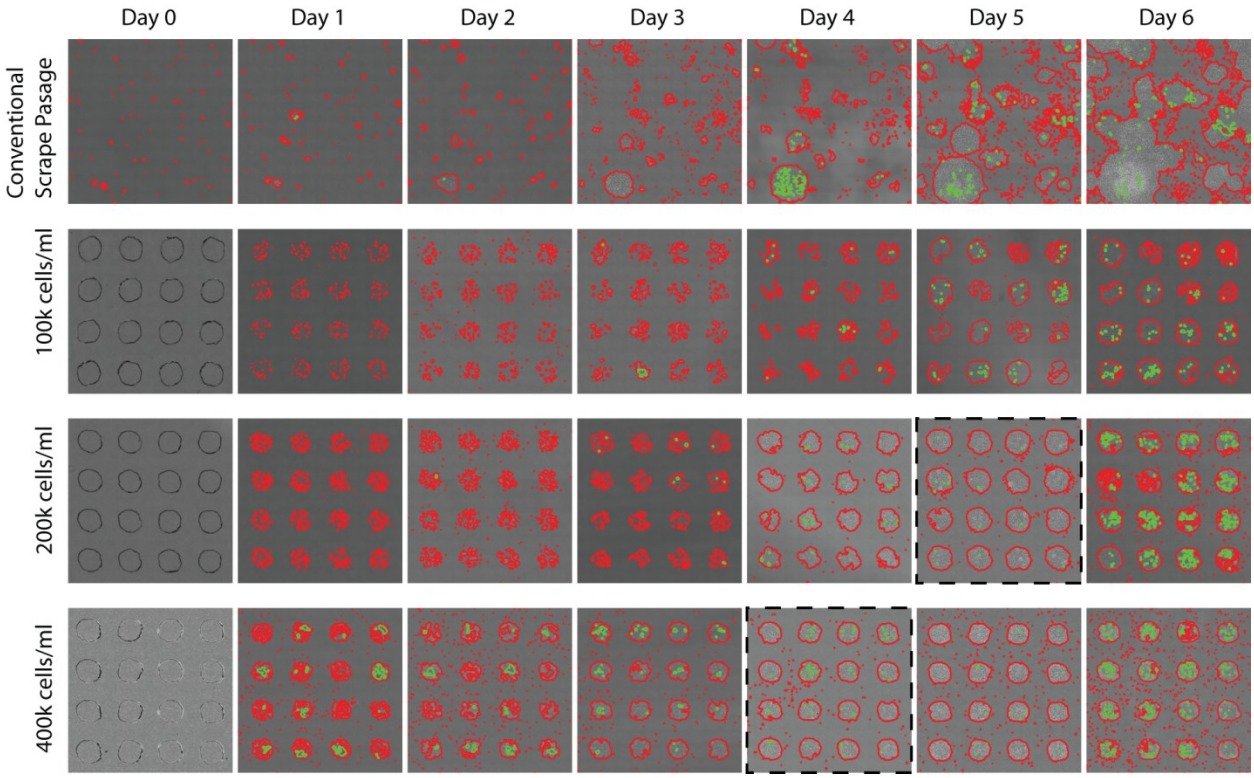
Supplementary Information

Suppl. Fig. 1: Cropped images from raw data used in immunofluorescence analysis.



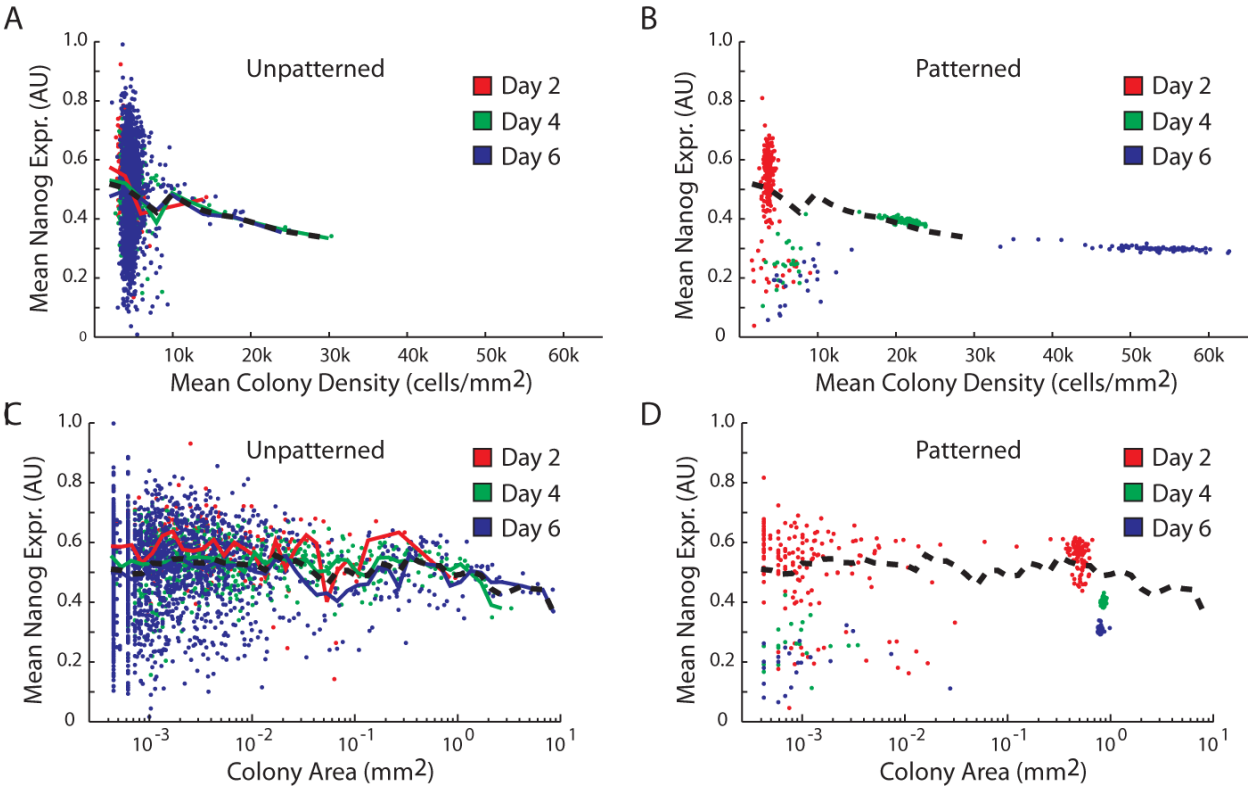
Raw immunofluorescence images of Nanog, SSEA4, and DAPI for D2, D4, and D6 of unpatterned and patterned cells. Merged images of Nanog and SSEA4 show a loss of pluripotency expression in dense regions in the unpatterned wells, whereas for patterned wells expression is very uniform. Ratiometric heatmaps, based on the ratio of protein to DAPI, also confirm this trend. Note that these cropped tiles show an area of about 7 x 7 mm, whereas the full image is 26 x 26 mm, including one hundred 1 mm colonies.

Suppl. Fig. 2: Seeding cells at different concentrations leads to tunable time-to-confluence and cell density.



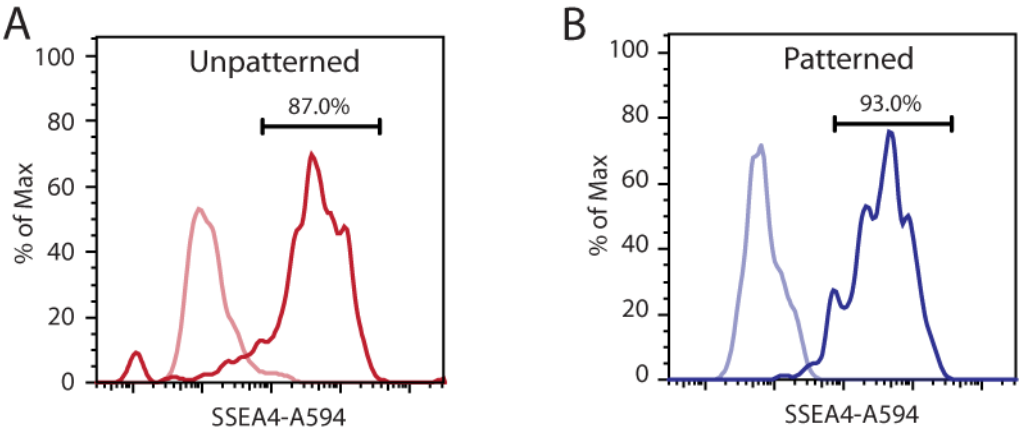
The above contrasts the substantial heterogeneity in unpatterned, conventional clump passage culture (first row) with the homogeneity in patterned single cell passage (second through fourth rows). Day-by-day phase-contrast microscopy mosaics shows a section of a 10 x 10, 1mm grid pattern of hiPSC cells at different initial seeding densities. Dashed boxes indicate timepoints at which colonies are completely filled in on the patterns. Note that the lower densities take longer to fill the patterns, whereas the high concentration (400k cells/ml) completely fills the patterns by day 4. The red outline indicates the outer perimeter of the colonies while the green indicates vacancies within the colonies. The vacancies are initially filled by dividing cells at early timepoints, but also occur at later timepoints as cells detach when cell density is too high.

Suppl. Fig. 3: Pluripotency marker expression vs. overall colony density and colony size



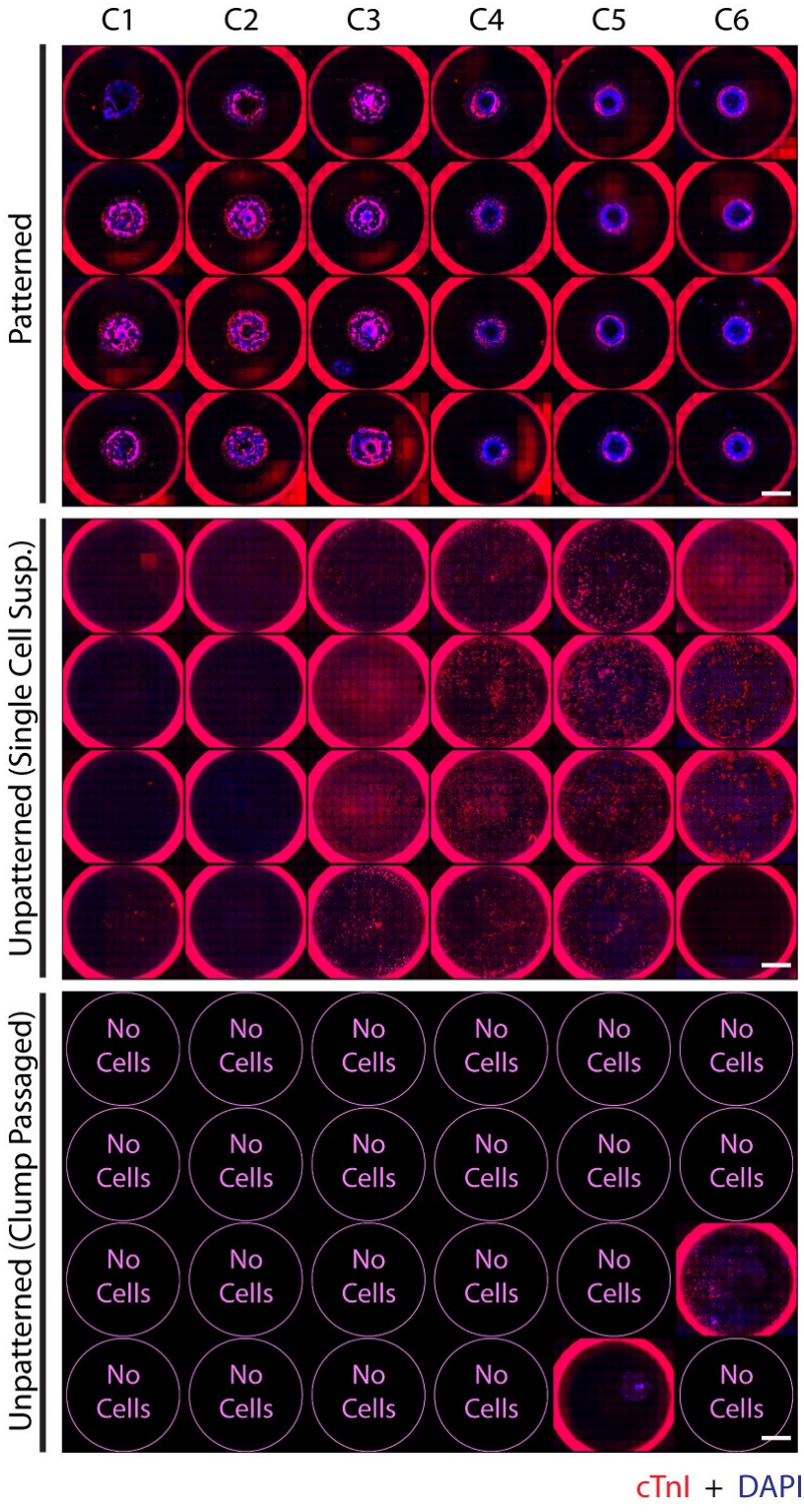
(A-B) In addition to depending on local cell density, Nanog expression is also a strong function of overall mean colony density, with denser colonies resulting lower overall Nanog expression. Patterned colonies are also much more tightly clustered in terms of density and Nanog expression. Note that this colony segmentation algorithm is not sensitive to sparse, nontouching cells, so in this data we do not see a similar decrease in pluripotency below 3k cells/mm^2 as we do in the previous analysis (Figure 4). (C-D) Mean Nanog expression is not a strong function of colony area.

Suppl. Fig. 4: Example of SSEA4 flow cytometry data



Overall SSEA4+ fraction at day 4 was not significantly higher in patterned cells versus unpatterned cells. Lighter line indicates isotype control signal. Gate thresholds were chosen such that <1% of the isotype control was counted positive.

Suppl. Fig. 5: All cultures at day 15, showing immunostaining for cTnI and DAPI counterstaining.



Suppl. Fig. 6: Cardiomyocyte differentiation conditions

	Day					
	D0	D1	D3	D5	D7	D9-15
C1	BMP-2.5	BMP-10 ActA-5 bFGF-5	IWR-10	-	-	Complete RPMI
C2	BMP-2.5	Same	IWR-5	-	-	Same
C3	BMP-0.5	Same	IWR-10	-	-	Same
C4	BMP-0.5	Same	IWR-5	-	-	Same
C5	BMP-2.5	Same	Dkk-37.5 SB-5 Dorso-0.5	Dkk-37.5	bFGF-5	Same
C6	BMP-0.5	Same	Dkk-37.5 SB-5 Dorso-0.5	Dkk-37.5	bFGF-5	Same

BMP = BMP-4

ActA = Activin A

bFGF= basic FGF

Dkk = Dkk-1

SB = SB431542

Dorso = Dorsomorphin

IWR = IWR-1

All units in ng/mL or µM, see text for details

Video 1: Spontaneous cardiomyocyte contractions at D14, differentiation condition C1 (well #1).

Video 2: Spontaneous cardiomyocyte contractions at D14, differentiation condition C2 (well #3).

Video 3: Spontaneous cardiomyocyte contractions at D14, differentiation condition C3 (well #2).

Video 4: Spontaneous cardiomyocyte contractions at D14, differentiation condition C4 (well #2).

Video 5: Spontaneous cardiomyocyte contractions at D14, differentiation condition C5 (well #2).

Video 6: Spontaneous cardiomyocyte contractions at D14, differentiation condition C6 (well #3).