Validation of label–free, real–time selection of fully reprogrammed iPSC colonies using kinetic image pattern recognition

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ABSTRACT

The ability to reprogram somatic cells to an embryonic stem cell-like state has had a landmark impact on basic biological research, drug screening, and drug discovery. However, picking fully reprogrammed induced pluripotent stem cell (iPSC) colonies can be unreliable, costly and time consuming. In particular, currently there are no methods to consistently and automatically pick fully reprogrammed iPSC colonies without fluorescent surface markers. Label free and automated selection of iPSC colonies would greatly reduce the complexity of automated reprogramming and expansion systems, save time and resources in iPSC production, and facilitate high-throughput application of iPSC technology. In addition, for researchers less familiar with iPSC technology, computer assisted iPSC colony selection would ease implementation of reprogramming in their laboratories.

We have discovered specific patterns of iPSC colony formation that can be used to predict whether they will become fully reprogrammed. We have developed software methods to quantify and score these colony formation patterns using phase contrast video microscopy, and predict which colonies will become iPSCs. Since, in general, only a few fully reprogrammed iPSC colonies per patient sample are needed, we designed our approach to achieve very high specificity while allowing lower sensitivity. We created the method using a development database of seven patient samples comprised of time-lapse recordings showing thousands of colonies forming, rigorous annotated ground truth on over 1200 colonies (TRA-1-60 staining and manual review), and pluripotency characterization of eight cell lines.

In the current study, we have validated the method in a double blinded study using seven healthy and disease-specific fibroblasts and Sendai virus-mediated reprogramming with Klf4, Oct3/4, Sox2 and c-Myc, imaged continuously for four weeks in the Nikon BioStation CT. The kinetic image pattern recognition software automatically identifies and ranks up to 10 fully reprogrammed colonies for selection per patient using only time-lapse phase contrast microscopy image sequences without fluorescence. Concurrently, skilled technicians in the HSCI iPSC core select fully reprogrammed colonies based on morphology and TRA-1-60 staining. Here we present the kinetic image pattern recognition method and validation results that show fully blinded and automated selection of iPSC colonies with high specificity. Pluripotency validation was performed and comparison between human and computer selected colonies shows no significant difference in colony quality using standard and rigorous pluripotency assessment methods.

IMAGING PROTOCOL

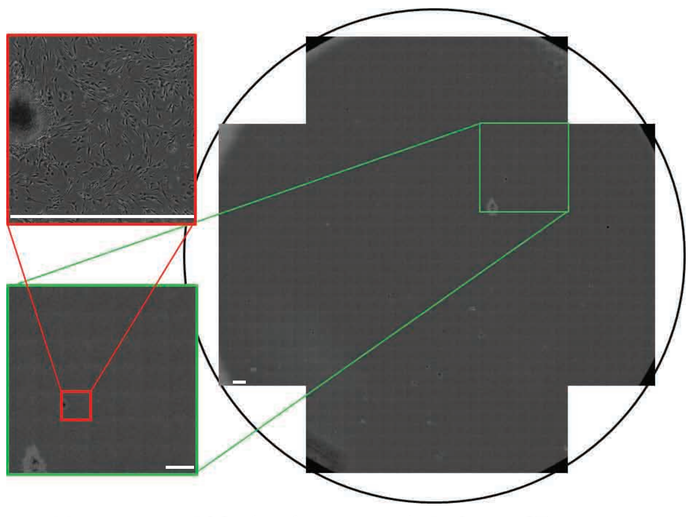

Figure 1. *Monitoring rare iPSC colony formation on the BioStation CT*
Our research was conducted using the Nikon BioStation CT and CL-Quant software (Nikon Corporation). The CT allows continuous imaging and incubation of the cells undergoing reprogramming. In these studies the CT generates spatially aligned images which are formed into a composite of tiled image sequences upon loading in CL-Quant covering the complete 10 cm dish. For the development image sets, one time point consists of 21, 7x7 image tilings as shown in the figure above (white scale bar = 2,000 µm). The format has changed slight for the current blind study; one time point consists of 16, 10x10 image tilings covering the entire dish at the same optical resolution (images not shown). The CT provides long term, whole vessel coverage enabling detection of the rare iPSC colony formation events. Patient samples used in this study (7 development samples and 7 blind test samples) include both healthy neonatal, adult and diseased samples from patients with Spinal Muscular Atrophy (SMA), Amyotrophic lateral sclerosis (ALS) and Parkinson's Disease.

KINETIC IMAGE PATTERN RECOGNITION

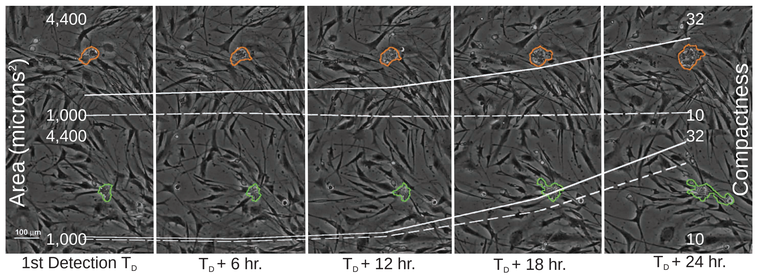

Figure 2. *Kinetic characterization of iPSC formation*
The detection of rare iPSC colony formation events is done using a kinetic image pattern recognition (KIPR) module. The steps include detection, tracking, quantitative characterization, filtering & classification, and scoring. Figure 2 shows area (solid line) and compactness (dashed line) metrics for a iPSC colony (orange outline) and non iPSC colony (green outline) in the first five frames (24 hours) in which they are detected.

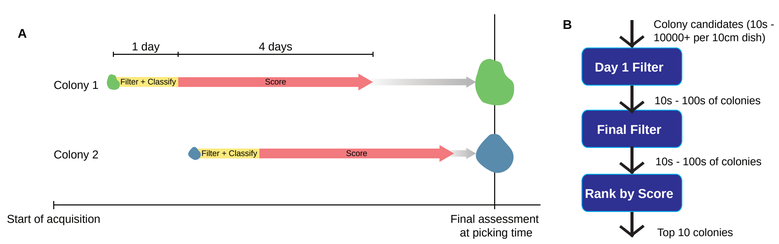

Figure 3. *Filtering, classification, and scoring of iPSC colonies for selection*
The vast majority of detected and tracked colony candidates are removed from consideration within the first 24 hours of their formation. After this Day 1 Filter, approximately 85% of the remaining candidates are TRA-1-60+ (data not shown). However, many of these colonies disaggregate during the third week post transduction. Therefore a final assessment and filtering at colony selection time is performed, removing colonies that are too small or have merged with other colonies. The remaining colonies are ranked automatically using the scoring rule using metrics from the first five days of imaging after each colony's formation. The top ten "machine" ranked colonies are provided to the user for picking. If there are less than 10 viable colonies, then only the top viable colonies are provided. Viability critiera can be set using a minimum score threshold.

RESULTS

		Ground Truth		
		Positive	Negative	
Test	Positive	TP	FP	
	Negative	FN	TN	
Test	Positive	92	8	100
	Negative	72	1098	1170
		164	1106	1270

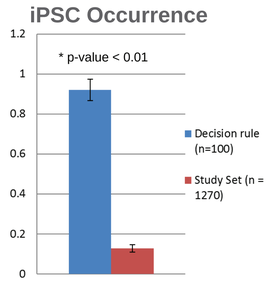

Figure 4. *iPSC classification accuracy verification*
Performance is evaluated using our development set of 7 patient samples, 147 tiling image sequences and 1,270 colonies with carefully annotated truth. 2x2 contingency table is shown at left (TP true positive, FP false positive, FN false negative, TN true negative). Roughly 30% of the development data was used for training and ~70% for verification. Results are reported for all data. Overall classification accuracy (~30% training, ~70% test) is 93.70 +/- 1.31% with specificity (proportion of non iPSC classified as non iPSC) of 99.28 +/- 0.50% (with sensitivity of 56.10 +/- 7.60%). As described above, the objective is to have near perfect specificity; lower sensitivity is acceptable. Significance of selection as compared to random is tested using a one-sided student's t-test and comparison of confidence intervals (CIs). As shown in the figure at right, the CI of true iPSC occurrence in our rule set (92.0+/- 5.32%) does not overlap with the CI of the entire study set (12.91+/-1.84%); the difference is highly significant with a p-value of 2.12x10-99.

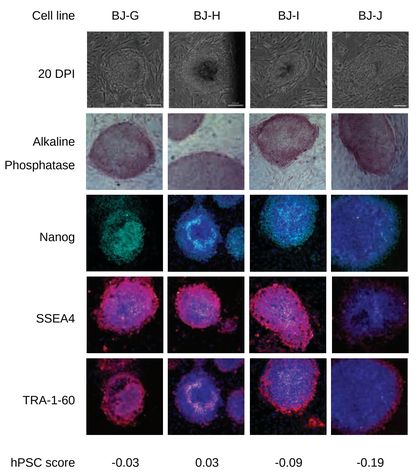

Figure 5. *Machine selection in blind study*
We assessed machine scoring in a double blinded study using a single sample of neonatal fibroblasts. KIPR processing was done in real time, and the machine's top ten colonies were provided and the top four were picked manually and expanded. In practical usage it is critical that the there be limited or no false positives in the machine's ranking. The results were an unequivocal success, with 4 of 4 of our selections judged to be fully reprogrammed iPSC colonies. Pluripotency is confirmed using immuno-staining of pluripotent protein expression (Oct4, Nanog, SSEA3, SSEA4 and TRA-1-60), and also using the Thermo Fisher hPSC scorecard (where a score from -0.5 to 0.5 indicuates pluripotency).

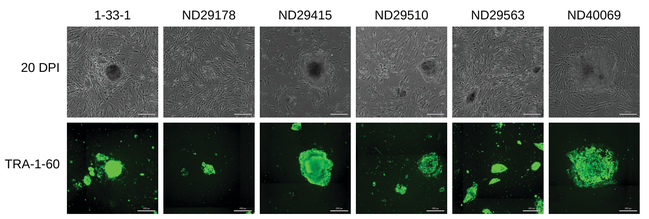

Figure 6. *Ongoing validation of machine selection performance*
Six additional patients have been reprogrammed (96 new sequences) and selected in a double blinded fashion, but full pluripotency characterization has yet to be completed. However, pluripotency was assessed with a TRA-1-60 live staining. For each patient, all of the viable (minimum score was set to 0.80 out of 1) machine-selected colonies are considered. We find that 55 of 58 machine selections are strongly TRA-1-60+, yielding a positive prediction rate of 94.83+/-5.7%. The figure shows the top machine selected colony for each new patient and associated TRA-1-60 live staining image.

	Sample Name	Self-renewal	Ectoderm	Mesoderm	Endoderm
Human-selected	BJ A ips	-0.05	0.06	0.32	-0.27
	BJ B ips	0.02	0.35	0.71	0.07
	BJ C ips	0.00	0.14	-0.57	-0.48
	BJ D ips	-0.13	-0.11	0.76	-0.36
Machine-selected	BJ G ips	-0.03	-0.37	-0.79	-0.31
	BJ H ips	0.03	-0.30	-0.44	-1.03
	BJ I ips	-0.09	-0.29	-0.26	-0.11
	BJ J ips	-0.19	-0.34	0.00	-0.38

Gene expression relative to the reference standard

Upregulated						Downregulated
x > 1.5	1.0 < x <= 1.5	0.5 < x <= 1.0	-0.5 <= x <= 0.5	-1.0 <= x < - 0.5	-1.5 <= x <-1.0	x < -1.5

Figure 7. *Comparison of human and machine selection*
Human-expert-selected lines (A,B,C,D) and machine-selected lines (G, H, I, J) are compared in the table at left using Thermo Fisher hPSC scorecard applied to stem cell lines to assess pluripotency quality. All colonies have appropriate self-renewal gene expression levels (-0.5 <= x <= 0.5). However, human-selected lines B ips and D ips are slightly upregulated for Mesoderm expression, but are still within the normal range. Based on this data we can say that the quality of machine selection, in terms of pluripotency markers and lack of upregulation of trilineage markers, is on par or superior to human selection.

DISCUSSION

These results strongly validate the machine-selection method for automated or user assisted selection of fully reprogrammed iPSC colonies. In the next step we will look to validate the machine-selection method with other reprogramming technologies such as episomal vectors and mRNA transfection. At the same time , we will integrate the KIPR module including classification and scoring with a real-time incubation and imaging system like the BioStation CT as a functional prototype for more extensive validation using Sendai viral-mediated reprogramming of fibroblast cells.

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