Video Article

Profiling Individual Human Embryonic Stem Cells by Quantitative RT-PCR

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Abstract

Heterogeneity of stem cell population hampers detailed understanding of stem cell biology, such as their differentiation propensity toward different lineages. A single cell transcriptome assay can be a new approach for dissecting individual variation. We have developed the single cell qRT-PCR method, and confirmed that this method works well in several gene expression profiles. In single cell level, each human embryonic stem cell, sorted by OCT4::EGFP positive cells, has high expression in *OCT4*, but a different level of *NANOG* expression. Our single cell gene expression assay should be useful to interrogate population heterogeneities.

Video Link

The video component of this article can be found at https://www.jove.com/video/51408/

Introduction

Most higher eukaryote populations are heterogeneous thus with analysis of pooled population, it is often difficult to interpret their cellular features. Individual cells within a population may be subtly different, and these differences can have important consequences for the property and function of the entire population[1,2]. Especially, human embryonic stem cells (hESCs) are known to be heterogeneous, which causes different levels of pluripotency and diverse potentials to lineage specification in delicately distinctive ways[3,4]. For example, different cell surface antigens can be used to categorize undifferentiated pluripotent stem cells,[5] and the Austin Smith group proposed different levels of pluripotency in mouse embryonic stem cells, based on their morphology, differentiation propensity and dependency of signaling pathway[6]. This phenomenon was hypothesized in human embryonic stem cells[7]. Whereas the overall studies were performed among different stem cell lines, not individual single stem cells, it could be very intriguing to analyze different levels of pluripotency at the single cell level, which potentially affects their differentiation capacities toward all somatic cell lineages.

Cellular and molecular heterogeneity could be dictated by transcription profiling, which is called the 'single cell transcriptome' and emphasizes new approaches for quantifying gene expression levels[8-10]. For analysis of gene expression levels in individual cells, we developed a simple, but robust protocol of single cell quantitative RT-PCR. We confirmed the efficacy and feasibility of our protocol by comparing each half of single cell lysates as well as serially diluted total RNAs of hESCs, resulting in minimal technical variations and differences. Further, we used a genetic reporter line to isolate homogenous population of hESCs using gene targeting system. The donor vector for targeting OCT4 locus (OCT4-2A-EGFP-PGK-Puro construct) and a pair of TALEN plasmids were used[11]. The donor vector and a pair of TALEN plasmids were introduced into hESCs (H9, WA09) using our nucleofection and clonal selection protocol and maintenance of hESCs was performed based on our routine protocol[12]. We confirmed this genetic reporter line express EGFP for OCT4 expression in *OCT4::EGFP* hESCs.

Our result demonstrates that individual hESCs (sorted by *OCT4::EGFP* strongly positive cells) hold high levels of *OCT4* expression, but different levels of *NANOG* expression. So, our single cell gene expression assay should be useful to study population heterogeneities of pluripotent stem cells.

Protocol

1. Preparation of a 96-well Plate

1. Mix 1 µl of Single Cell DNase1 to 9 µl Single Cell Lysis Solution.
2. Put the 10 µl mixed solution in each well of 96-well PCR plate.

2. Detaching hESCs for FACS Purification

1. Detach *OCT4::EGFP* ES cell line from the 60 mm dish with 1 ml Accutase for 20 min, at 37 °C, which were neutralized with human ES media.

2. Prepare cell population in 1 ml FACS buffer and adjust the cell to 1 x 10^6 cells/ml.
3. Pass the cell sample through a 35 µm cell strainer cap tube.
4. Store the tube in ice before cell sorting.

3. Lysis of FACS-purified Single Cell in Each Well of the 96-well Plate

1. Sort the sample for EGFP positive cells on a cell sorter with a trained operator. Put the single cell into Single Cell Lysis/DNase1 solution in 96-well PCR plate. If necessary, the 96-well plate with sample can be stored in a -80 °C deep freezer less than one month.
2. Incubate samples 5 min at RT for cell lysis.
3. Add 1 µl of Stop Solution to stop lysis reaction.
4. Incubate 2 min at RT.

4. Reverse Transcription

1. Add to each a 0.5 µl aliquot of 20 µM SMA-T15, SMA-A.
2. Add 4 µl 5X buffer, 2 µl DTT, 1 µl Reverse Transcriptase, and 1 µl dNTP to each well.
3. Perform reverse transcription in a thermal cycler.
    1. Set the thermal program at 42 °C × 90 min and inactivate Reverse Transcriptase at 85 °C × 5 min.

5. Amplification

1. Add 4 µl of ExoSAP-IT reagent to each reverse transcribed sample.
    1. Incubate samples at 37 °C for 15 min and 80 °C for 15 min to inactivate the ExoSAP-IT reagent.
2. Prepare PCR reaction mix with SMA-p2 (2 nM)
3. Add 10 µl of PCR reaction mix to each reverse transcribed sample.
4. Perform the amplification, consisting of 20 cycles of denaturation (94 °C for 30 sec), annealing (57 °C for 30 sec), and extension (68 °C for 10 min).

6. qRT-PCR Performance

1. Add 10 µl of 2X SYBR Green PCR Master Mix, 1 µl amplified cDNA, 2 nM primers, and 7 µl water to each well.
    1. Set the program followed by 95 °C for 3 sec, 60 °C for 30 sec x 40 cycles.
2. Perform in duplicate for technical errors.

Representative Results

Efficient and robust single cell RNA amplification

To minimize the transcriptional variation among hESCs, we used *OCT4::EGFP* hESC clone for FACS purification. After sorting *OCT4::EGFP* positive cells into a 96-well plate, each cell is lysed in lysis buffer and converted poly(A)+ RNA to full length cDNA using SMA-T15 (GACATGTATCCGGATGTTTTTTTTTTTTTTTT) primer and anchoring with SMA-A (ACATGTATCCGGATGTGGG) by using SMART template switching technology. The excess oligonucleotides were digested with ExoSAP-IT reagent, then followed by 18-20 cycles of PCR amplification of cDNA with SMA-p2 (GACATGTATCCGGATGT)[13]. We used the amplified cDNA to make the template for qRT-PCR (**Figure 1**). There are several studies for full length RNA sequencing and measurement of RNA variability by using low quantities of cells and single cells[3,14,15]. To our knowledge, we diluted total RNA of hESCs (microgram amounts) down to nano- and pico- gram levels and applied our protocol to assess technical variability and detection of difference on low amounts of total RNA. We determined the reproducibility in gene expression levels generated from diluted RNA and individual cells. Analysis of the diluted RNA serially shows correlation among each sample and qRT-PCR results with several single cells show the similar Ct values in *GAPDH* gene (**Figure 2**).

Quantitative assessment of single cell gene profiles

To validate consistency among different batches of reverse transcription reactions, we divided FACS-purified single cell lysates into half and applied our protocol separately to each half of lysates for batch comparisons. We sorted strong EGFP positive cells by using FACS sorter (**Figure 3**), so *OCT4* expression level was high in EGFP positive cells, but *NANOG* expression level is various. The result shows significant correlation between the *OCT4* Ct value of each half of single cell lysates (**Figure 4**).

Analysis of embryonic stem cell gene profiles

We sorted individual hESCs and analyzed their gene expression level using our protocol, which shows consistent level of *OCT4* expression, and then we checked another stem cell marker gene *NANOG* in hESCs. As a result, *OCT4* gene expression level was high in every single cell, but *NANOG* shows different patterns (**Figure 5**).

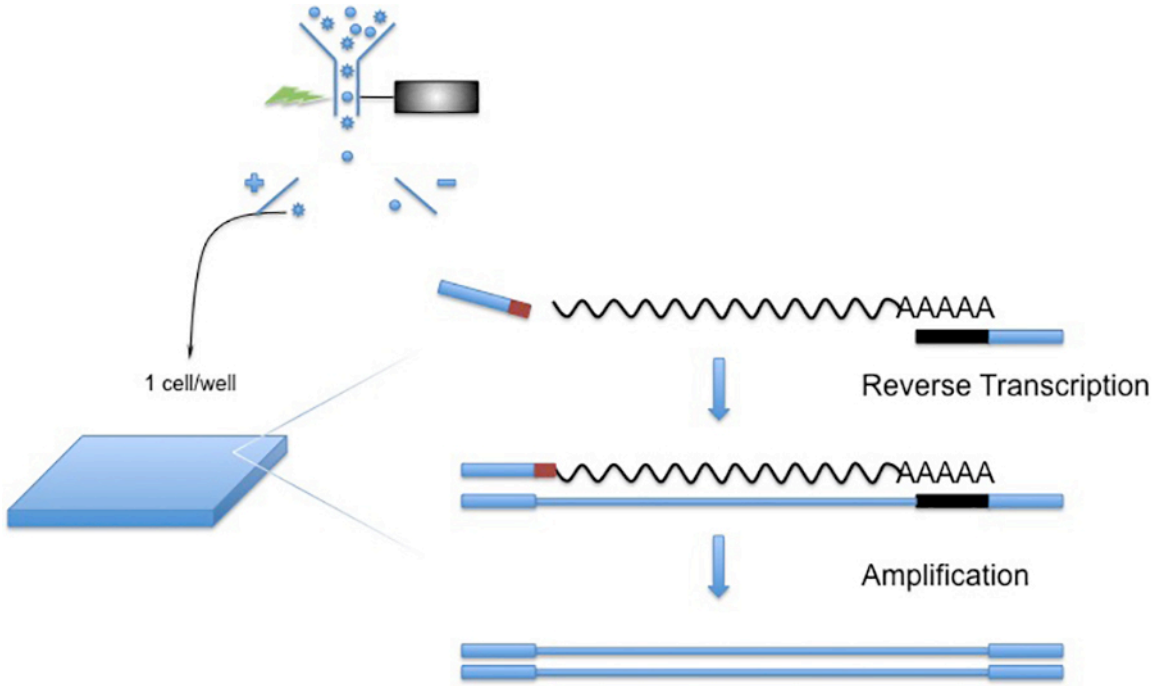

Figure 1. Schematic overview of single human embryonic stem cell qRT-PCR after FACS purification. Individual EGFP positive cells are sorted into each well of a 96-well plate containing cell lysis buffer. Lysed single cell went through reverse transcription with RTase. Remaining nucleotides are cleaned up using SAP/EXO, then product is amplified using PCR reaction with Taq DNA polymerase.

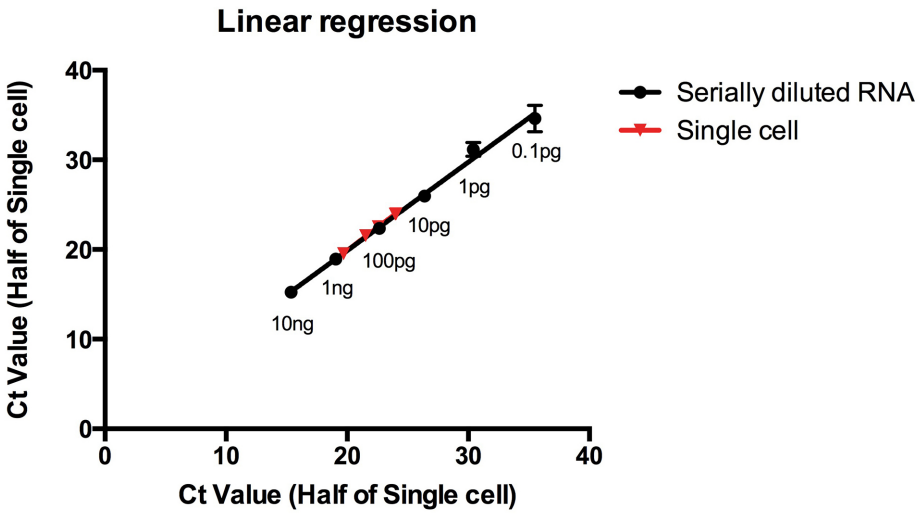

Figure 2. Real-time RT-PCR of *GAPDH* using serially diluted mRNA of pooled human embryonic stem cells. Each dot shows the Ct value of serially diluted mRNA and single cell mRNA sorted by FACS. Total RNAs were diluted from 10 ng/ul to 0.1 pg/ul. We repeated same experiments for comparison and made linear plot.

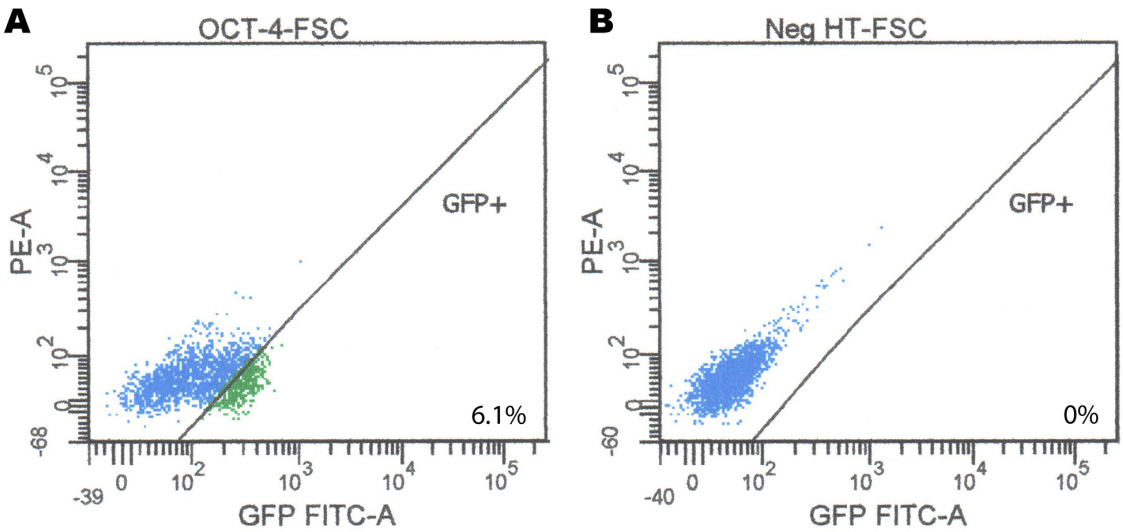

Figure 3. FACS analysis of OCT positive cells. We sorted EGFP positive cell by using a FACS sorter. *OCT4::EGFP* reporter hESC line shows around 60% EGFP positive population compared with negative population **(B)**. We selected strong EGFP positive cells **(A)**.

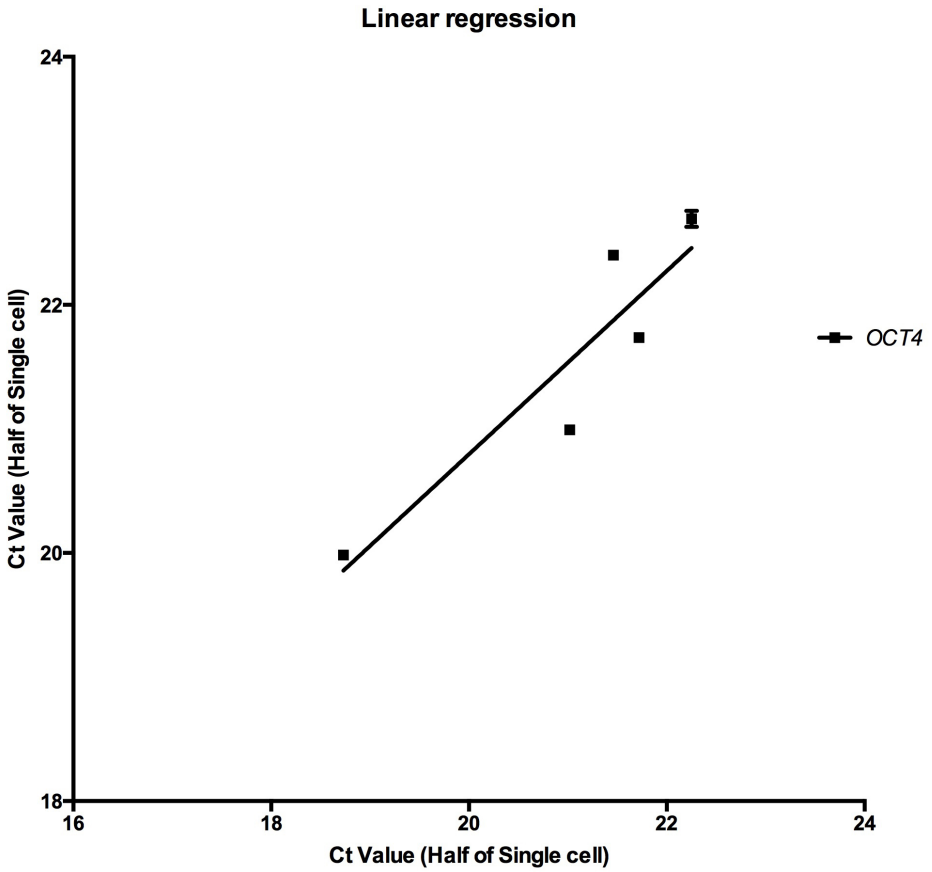

Figure 4. Comparison of gene expression level in each half of single cell lysates. To validate consistency among different batches of reverse transcription and amplification reactions, we divided single cell lysates in half, and applied our protocol for batch comparison.

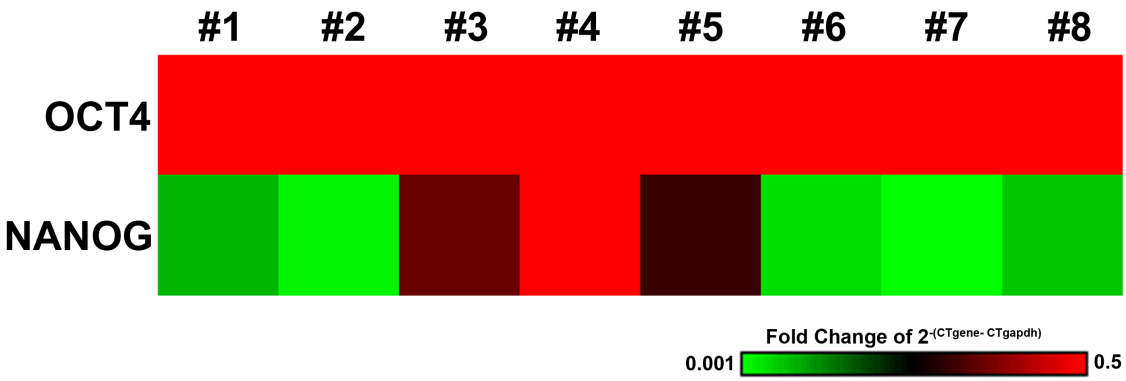

Figure 5. Heat map presentation of single human embryonic stem cell gene expression. Single cell gene expression analysis purified by FACS shows robust expression in *OCT4*, but different expression level in *NANOG*.

Discussion

Single cell gene profiling could be a major tool to predict functionality of a single cell or an entire population. Due to technical limitation, whole gene profiling analysis has been restricted to population averages. Variations in gene expression patterns and levels between individual cells and the subpopulations have been proposed to cause erroneous interpretation. Such diverse cellular aspects can be found in hESCs and their heterogeneity causes subtly different ability for maintaining pluripotency and fate specification process.

We developed and validated for single cell quantitative RT-PCR, which provides simple, but robust method for gene expression study in individual cells. To validate our protocol, lysates of FACS-purified single cell was divided into half and applied to this method for checking accuracies. Our result with single cells was corroborated with serially diluted total RNA samples and we found consistent results between each half of cell lysate (**Figure 4**). However, with our protocol, there are a few limitations. We could detect expression level of over 40 genes, but specific transcription information (*e.g.* non-polyadenylated mRNAs, miRNA, unknown transcripts, etc.) may not be detectable. Also, there are some opportunities to optimize the primers for target genes. The accepted primer length is usually 18-22 base pairs. The annealing temperatures of primers generally work in the range of 50-60 °C. The GC contents of primer can be affected by the annealing ability of the primer. In this paper, we removed the annealing step in qPCR to reduce the error of primer annealing. We only showed the *GAPDH* gene as a control, but the other house keeping genes can be useful as a control. Currently we are optimizing our protocol for RNA sequencing approach, which will uncover more detailed transcriptome of isolated single cells in near future. The other limitation is accuracy of FACS purification for isolating single cells. FACS machine mostly allocates single cells into each well, but we cannot exclude the possibility that each well may contain more than one cell, which can be figured with technical advance of FACS purification system. Nevertheless, our protocol can be directly applicable to any lab with minimal efforts and cost effective way for profiling single cell gene expression.

As shown in **Figure 5**, *OCT4::EGFP* positive single cells by sorted FACS maintain similar level of *OCT4* gene expression, but *NANOG* showed diverse expression pattern, which might suggests the different status of pluripotency of hESCs or reprogramming process[1]. One can profile other pluripotency gene expression in single cells of hESCs using different cell surface markers (TRA-1-60, SSEA3, ICAM1, CD44), and/or genetic reporter system (NANOG, REX1, STELLA, ESRRB, etc.)[5-7]. By using this protocol, single cell gene expression approach is easy and powerful method for heterogeneous populations such as hESCs or other types of stem cells.

Disclosures

The authors have nothing to disclose.

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References

1. Miyanari, Y., & Torres-Padilla, M. E. Control of ground-state pluripotency by allelic regulation of Nanog. *Nature.* **483**, 470-473, doi:10.1038/nature10807 (2012).
2. Leitch, H. G. *et al.* Embryonic germ cells from mice and rats exhibit properties consistent with a generic pluripotent ground state. *Development.* **137**, 2279-2287, doi:10.1242/dev.050427 (2010).
3. Ramskold, D. *et al.* Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. *Nat Biotechnol.* **30**, 777-782 (2012).
4. Stewart, M. H. *et al.* Clonal isolation of hESCs reveals heterogeneity within the pluripotent stem cell compartment. *Nat Methods.* **3**, 807-815 (2006).

5. O'Malley, J. *et al.* High-resolution analysis with novel cell-surface markers identifies routes to iPS cells. *Nature.* **499**, 88-91, doi:10.1038/nature12243 (2013).
6. Nichols, J., & Smith, A. Naive and primed pluripotent states. *Cell Stem Cell.* **4**, 487-492, doi:10.1016/j.stem.2009.05.015 (2009).
7. Kim, H. *et al.* miR-371-3 expression predicts neural differentiation propensity in human pluripotent stem cells. *Cell Stem Cell.* **8**, 695-706, doi:10.1016/j.stem.2011.04.002 (2011).
8. Mortazavi, A., Williams, B. A., Mccue, K., Schaeffer, L., & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods.* **5**, 621-628 (2008).
9. Pan, Q., Shai, O., Lee, L. J., Frey, J., & Blencowe, B. J. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet.* **40**, 1413-1415 (2008).
10. Wang, E. T. *et al.* Alternative isoform regulation in human tissue transcriptomes. *Nature.* **456**, 470-476 (2008).
11. Hockemeyer, D. *et al.* Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol.* **29**, 731-734, doi:Doi 10.1038/Nbt.1927 (2011).
12. Lee, G., Chambers, S. M., Tomishima, M. J., & Studer, L. Derivation of neural crest cells from human pluripotent stem cells. *Nat Protoc.* **5**, 688-701, doi:10.1038/nprot.2010.35 (2010).
13. Zhu, Y. Y., Machleder, E. M., Chenchik, A., Li, R., & Siebert, P. D. Reverse transcriptase template switching: A SMART (TM) approach for full-length cDNA library construction. *Biotechniques.* **30**, 892-897 (2001).
14. Pan, X. H. *et al.* Two methods for full-length RNA sequencing for low quantities of cells and single cells. *P Natl Acad Sci USA.* **110**, 594-599 (2013).
15. Tang, F. C. *et al.* RNA-Seq analysis to capture the transcriptome landscape of a single cell. *Nat Protoc.* **5**, 516-535 (2010).

