Rapid and Ultra-Sensitive Single-Cell Transcript Profiling with Droplet Digital PCR (ddPCR™): Application to neuronal differentiation

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Abstract

Single-cell transcript profiling is undoubtedly the ideal approach for gene expression analysis in neuronal tissues composed of various cell types. However, the robust detection of transcripts in isolated single cells or cytoplasm samples is technically challenging, especially without pre-amplification. Droplet Digital PCR (ddPCR) developed at Bio-Rad's Digital Biology Center directly counts individual molecules with superior precision and reproducibility. The ddPCR-based single-cell gene expression protocol measures even very low abundance transcripts with minimal sample processing for defined targets. Furthermore, ddPCR is performed in 96-well plates and is well suited to high throughput studies of focused sets of genes in large numbers of single cells.

In this work, we demonstrate the single-cell gene expression analysis of in vitro differentiated neuronal cells. We present a simple and robust workflow for profiling multiplexed, transcript targets in flow-sorted, neuronal single cells. We characterize a panel of validated assays targeting stem cell, proliferation and differentiation marker genes including nanog, p21, and Synaptophysin, respectively. We compare expression levels of these genes in non-differentiated versus differentiated single cells and bulk RNA preparation from the same cell populations prior to sorting. We demonstrate that ddPCR provides absolute counts of transcripts from >100,000 copies to <10 copies per cell. Our findings are discussed with current data in the literature.

Fig. 1: Single cell ddPCR work flow

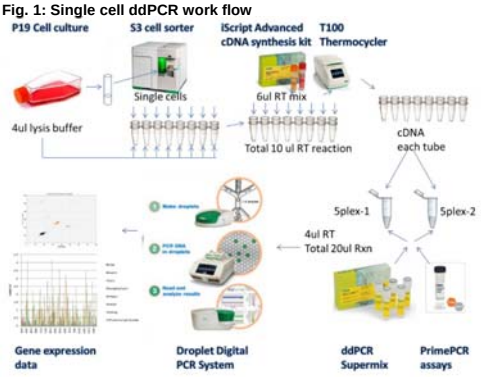

Fig. 2: P19 cell differentiation system

Modified from H.J. Monzo et al. *(2012)*

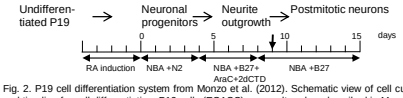

Fig. 2. P19 cell differentiation system from Monzo et al. (2012). Schematic view of cell culture conditions and timeline for cell differentiation. P19 cells (ECACC) were cultured as described in Monzo et al. with the following modifications: in vitro differentiation was performed on poly-L-ornithine (Sigma) and laminin (Life Technologies) coated dishes with coating performed per manufacturer's recommendations; cells were treated with AraC and 2dCTD from day 4 to day 8. RA, retinoic acid (at 1uM); NBA, neurobasal media; AraC, Cytosine -d-arabinofuranoside (at 8uM); 2dCTD 2´-Deoxycytidine (at 8nM).

Fig. 3: Multiplexing ddPCR data

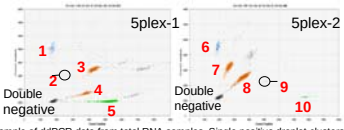

Figure 3: Example of ddPCR data from total RNA samples. Single positive droplet clusters are labeled with respective gene number (Table 2). Blue labeled droplets are from FAM-only assays; green labeled droplets are from HEX-only assays; orange labeled droplets are from mixed assays (FAM and HEX at various ratios, see table 1). Non labeled droplets represent double-or triple-positive droplets (only seen for very high concs).

Table 1: Multiplexing scheme

Gene	FAM (%)	Hex (%)	uL of assay per ddPCR rxn
1, 6	100	0	1.5
2, 7	75	25	1
3, 8	50	50	1
4, 9	25	75	1
5, 10	0	100	1.5

Table 1: Multiplexing strategy for simultaneous detection of five genes in one ddPCR reaction by using different amount of FAM and HEX PrimePCR assays (Bio-Rad). Two five-plex panels were assembled to measure expression of a total of 10 genes per single-cell.

Table 2: Gene panel of mouse PrimePCR Taqman assays (Bio-Rad)

Gene	#	Official name	Design type	Amplicon length	Marker, cell type
Nanog	9	Nanog homeobox	Intron-sp.	113	Pluripotency, undifferentiated embryonic stem cells
Sox2	4	SRY-box containing gene 2	exonic	103	
Nes	3	Nestin	Intron-sp.	60	Multipotent neural stem cells
Mki67	8	Antigen identified by monoclonal antibody Ki-67	exonic	102	Proliferating cells
Cdkn1a	6	Cyclin-dependent kinase inhibitor 1A (P21)	exonic	113	Cell cycle withdrawal, non proliferating cells
Mtap2	7	Microtubule-associated protein 2	Intron-sp.	155	Neuron-Restricted Progenitors and neurons
Chat	2	Choline acetyltransferase	Intron-sp.	118	Cholinergic neurons
Syp	5	Synaptophysin	Intron-sp.	113	Neurons and neuroendocrine cells
Th	10	Tyrosine hydroxylase	Intron-sp.	64	Dopaminergic neurons
Gfap	1	Glial fibrillary acidic protein	Intron-sp.	107	Astrocytes, neural progenitors

Table 2: Gene Panel of 10 marker genes for pluripotency, proliferation and differentiation from mouse PrimePCR assays (Bio-Rad). Assay design type and amplicon length are indicated. Intron-sp., Intron-spanning. # indicates numbering in ddPCR plots in Figure 3.

Methods

Reverse transcription from total RNA

Total RNA was extracted from P19 cells using the Aurum kit and reverse transcribed with the iScript™ Advanced synthesis kit (both from Bio-Rad). 1ng of reverse transcribed total RNA was subjected to ddPCR analysis.

Single cell Reverse Transcription

Cells were trypsinized, washed and resuspended in PBS with 0.25% BSA and 1mM EDTA. Cells were filtered through a 70u strainer and sorted using the S3™Cell Sorter (Bio-Rad). Cells were identified and gated using forward and side scatter plot. Doublet discrimination using the forward scatter width vs. height parameters was used to avoid collection of cell clusters. The cells were dispensed into 8 well strip tubes containing 4ul chilled lysis buffer (IDTE, pH 8, Rnase inhibitor, 0.1% Triton-X100). RNA in the lysate was reverse transcribed with the iScript Advanced synthesis kit in a total volume of 10ul (0.5ul RT, 2ul buffer). 4 ul of RT reactions were analyzed per 20ul ddPCR reaction.

5plex ddPCR

ddPCR reactions were assembled with ddPCR™ Supermix for Probes (no dUTP) and up to five assays with the multiplexing scheme shown in Table 1. PCR reactions were cycled at 55ºC annealing temperature.

References

Hector J. Monzo, Thomas I.H. Park, Johanna M. Montgomery, Richard L.M. Faull, Mike Dragunow, Maurice A. Curtis (2012). A method for generating high-yield enriched neuronal cultures from P19 embryonal carcinoma cells. Journal of Neuroscience Methods 204 (2012) 87– 103.

Fig. 4: Expression level in total RNA from P19 cells

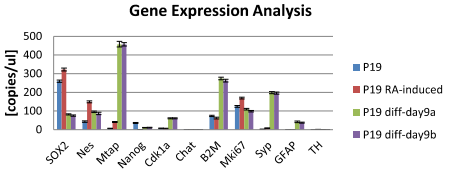

Figure 4: ddPCR gene expression analysis of total RNA samples. Data are from Duplex assays.

Fig. 5: Gene Expression in single P19 cells

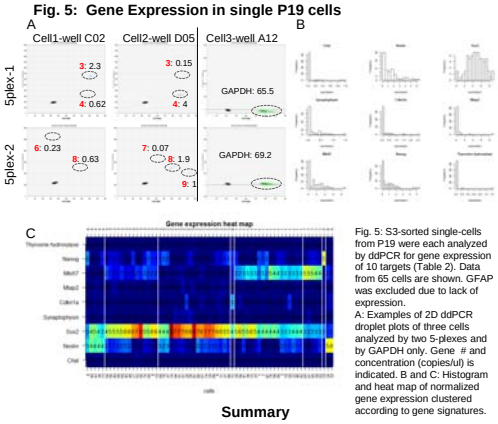

Fig. 5: S3-sorted single-cells from P19 were each analyzed by ddPCR for gene expression of 10 targets (Table 2). Data from 65 cells are shown. GFAP was excluded due to lack of expression.
A: Examples of 2D ddPCR droplet plots of three cells analyzed by two 5-plexes and by GAPDH only. Gene # and concentration (copies/ul) is indicated. B and C: Histogram and heat map of normalized gene expression clustered according to gene signatures.

Summary

Methods

- We established a multiplexing strategy for the simultaneous detection of five genes in a single ddPCR reaction using PrimePCR assays.
- We demonstrate a single-cell workflow for gene expression analysis of up to 10 genes per cell in two ddPCR reactions, without cDNA pre-amplification.

Biological Findings

- Differentiated P19 (day9) cells show up-regulation of neuronal markers (Mtap2, Syp), glial marker (GFAP) and down-regulation of stem cell markers (Sox2, Nanog), in concordance with literature (Monzo et al.).
- Single-cell analysis of P19 shows distinct cell types based on differential Sox2, Mki67 and Nestin expression.

Single-cell analysis of differentiated P19 is in progress.