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White Paper

# Single Cell Gene Expression Analysis with a Spectral Flow Cytometer

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## 1. Introduction

Embryonic stem cells (ESCs) provide an unprecedented opportunity for the study of human tissue development, and the development of cell-based therapies for human disease. To realize these potential advances, methods for monitoring the expression of intracellular proteins in live stem cells without altering cellular properties are needed. Molecular beacons are single-stranded oligonucleotides that have been employed to assay gene expression. To enhance gene detection specificity, we developed a dual fluorescence resonance energy transfer (FRET) molecular beacon (MB) system for detection of target gene expression by flow cytometry (King, *et al.* (2010) *Stem Cells Devel.*).

We demonstrated that expression of specific genes can be detected by FRET using confocal microscopy, that this methodology could be applied in a high throughput manner to the identification of target-expressing cells by conventional fluorescence-activated cell sorting (FACS), and that stem cells transfected with MBs demonstrate normal growth rates and oligonucleotide extinction over time. These studies demonstrated that dual FRET MBs provide a useful tool for identifying specific types of stem cells without functional or genomic alteration.

However there can be problems as some stem cell types display high autofluorescence levels that interfere with the detection of low expression genes. The dual-FRET MB system limits dye selection possibilities to specific small molecular weight FRET dye pairs. These FRET dye pairs often cover a larger range of the color spectrum than single probes, making it difficult to expand the experiment to more markers and other fluorochromes. In this report, we introduce the unique capabilities of the spectral flow cytometer developed by Sony Corporation to provide higher signal-to-noise ratios, by unmixing background signals including cell autofluorescence and multiple fluorochromes with overlapping emission signals. Spectral flow will eventually enable single cell gene expression analysis with multiple MB probes without the need for dual-FRET.

## 2. Background signals

There are two kinds of noise contained in detected flow cytometric signals. One is cell autofluorescence, and the other is the background luminescence independent of cells.

Figure 2A shows a spectrum chart of the measurement results from 8-peak Rainbow Calibration particles (RCP; Spherotech, Inc.), excited with a 488nm laser. Spectrum charts show wavelength in the horizontal axis, intensity in the vertical axis and density in color scale. We can see eight spectrum lines correspond to eight peaks of the RCP sample. A small bump in intensity around 580nm is found in the dimmest population. To investigate this bump, we measured the background luminescence under four conditions; no flow cell chip in the light path (No chip), flow cell chip without any liquid (Air), flow cell chip filled with Sheath liquid (Sheath) and D.I. water (Water) (Figure 3).

As a result, a similar bump around 580nm is found in Sheath and Water samples only, not observed in the chip-alone sample. When excited with a 488nm laser, the estimated Raman Scattering from water is around 584nm. This number matches with the bump we observed. We concluded that the bump is derived from Raman Scattering of water filled in the flow cell chip. While Raman Scattering is expected to occur in constant frequency with constant spectrum, we can estimate and subtract it from the detected signals. Figure 2B shows the RCP sample measurement result with Raman Subtraction calculation. The small bump around 580nm disappeared, and the spectrum looks much smoother. We applied this calculation in all analyses below.

Next we observed the cell autofluorescence signals. Figure 4 shows a spectrum chart of unstained ESCs samples. Here we can see deviation of the intensity of cell autofluorescence was up to $10^2$. In conventional multi-color analysis algorithms, average intensity of cell autofluorescence was subtracted from all events, and the deviation of the autofluorescence was not considered. The spectral flow cytometer has the potential to distinguish and unmix cell autofluorescence from other dye signals by its spectral feature, without neglecting the deviation of the intensity. In the following analyses, we show how we can spectrally unmix cellular autofluorescent signals to lower the background noise.

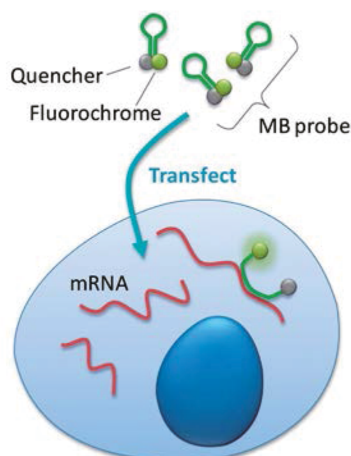


Figure 1. Molecular Beacon (MB) probe

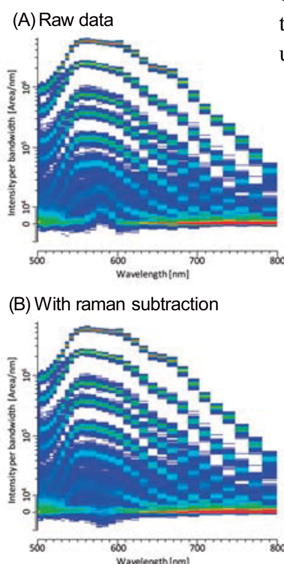


Figure 2. Rainbow 8 peak beads

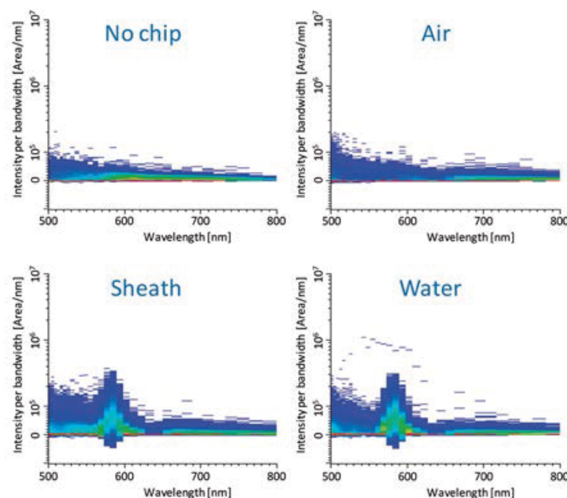


Figure 3. Background luminescence observation

## 3. Observing single cell gene expression

To evaluate single cell gene expression measurements, we prepared two MB probes both designed to target on Oct-4 mRNA, labeled with 6FAM/BHQ1 and 5ROX/BHQ2 at the ends of each probe. At basal condition, they are folded and fluorochromes (6FAM and 5ROX) are located adjacent to quenchers (BHQ1 and BHQ2), thus fluorescent signal could not be observed. Once the oligos are transfected into cells and bound to target mRNA molecules, the MB probes become "stretched" to set fluorochromes apart from quenchers and emit luminescence upon excitation (Figure 1).

ESCs were transfected with MB probes with 6FAM/BHQ1 (FAM) and 5ROX/BHQ2 (ROX), and measured with the spectral flow cytometer with 488nm laser excitation. Figure 5 shows spectrum charts of untreated and transfected ESCs. By comparing untreated and FAM samples, FAM dependent fluorescence could be recognized. That spectrum shape is very similar to cell autofluorescence, and the intensity is not largely different from deviation range of the autofluorescence. ROX signal is hardly distinguished by eyes because its excitation with 488nm is limited. We performed a spectrum compensation calculation based on a least square method taking into account cell autofluorescence, and presented scatter plots with FAM and ROX axes (Figure 6). Results showed clear separation of Oct-4 positive and negative populations in FAM stained samples. Also in ROX stained samples, MB dependent signals were confirmed in comparison with the untreated sample.

## 4. Conclusion

We have confirmed the spectral flow cytometer can successfully remove background noise derived from cellular autofluorescence and water derived Raman Scatter. This makes it possible to detect FAM signals without using dual-FRET MB even though the spectra of FAM and autofluorescence were very similar. The resulting increase in sensitivity provided by the spectral cytometry technology allows the use of single MB probes without FRET, and expands the number of gene targets that can be evaluated in a single experiment. In summary, we confirm that the spectral flow cytometer provides high sensitivity even when the luminescent spectra of signal and background are very similar, and demonstrates a unique capability to analyze cellular spectra at the single cell level. We believe this cytometer has the potential to accelerate single cell gene expression analysis that in turn impacts all aspects of cell biology.

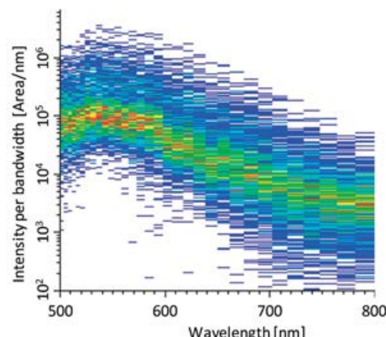


Figure 4. ESCs autofluorescence

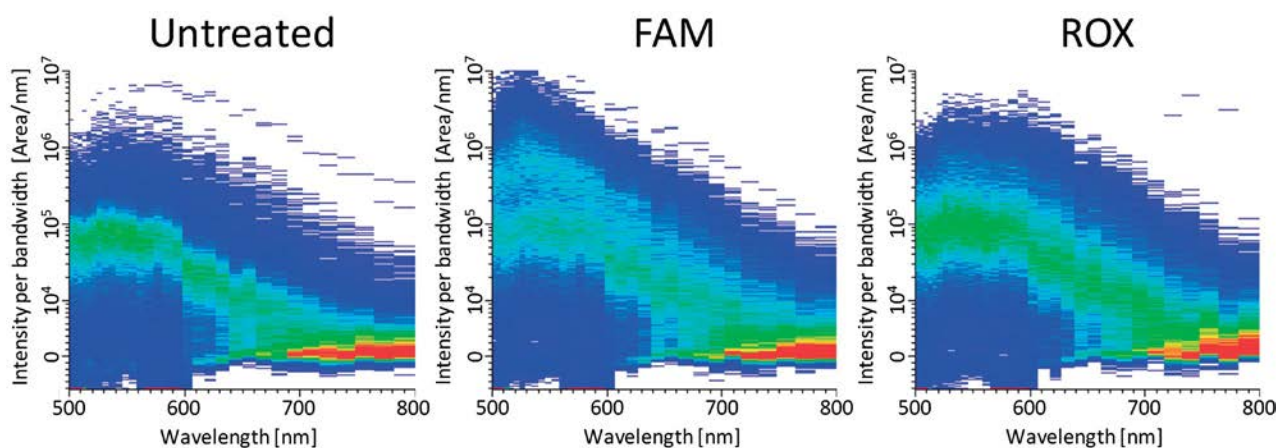


Figure 5. Spectrum charts of ESCs stained with MB probe

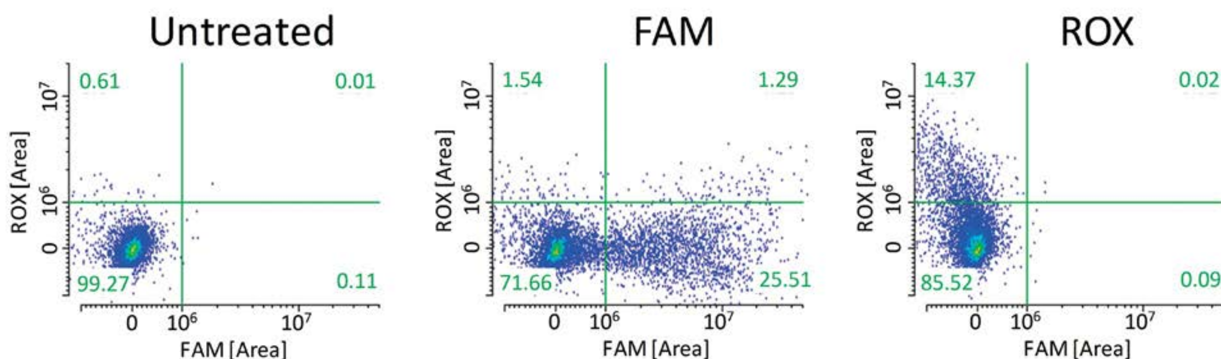


Figure 6. Scattering charts of ESCs stained with MB probe


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