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Application Note

SINGLE CELL ANALYSIS OF RNA FISH FOR CYTOKINE EXPRESSION

Background

Some cell types exhibit significant heterogeneity which can limit resolution when analyzing gene expression with techniques such as qPCR. In recent years the development of single molecule RNA FISH has enabled the detection of RNA expression at the single cell level, however traditional confocal imaging lacks sufficient throughput to investigate smaller subsets of cell populations. RNA FISH technology also provides an opportunity to visualize expression of targets which are difficult to stain at the protein level via immunofluorescence.

Hermes High content wide field microscope offers the ability to work with large sample sizes while investigating subcellular features at the single cell level. This technique produces large numbers of images which necessitate automated computational analysis. Quantitation of features per individual cell can provide new insights into cell biology.

In this application note, we utilized *WiScan® Hermes* high content microscope to investigate immune responses to stimuli by primary human monocyte-derived macrophages, which exhibit significant variability in gene expression between different cells.

Materials & Methods

Day 7 human monocyte-derived macrophages (MDMs) were stimulated with the innate immune agonists Zymosan, LPS or Poly I:C for 4 hours and then fixed (Figure 1). *ViewRNA FISH* kits (Thermo Fisher) were used to stain Tumor Necrosis Factor Alpha (TNF-a) and Interleukin-10 (IL-10) mRNA as markers of pro-inflammatory and regulatory signaling, respectively. Cells were imaged using a *Hermes Wiscan®* (IDEA Bio-Medical) high content microscope prior to quantitation using Metamorph 7 (Molecular devices) and R.

RNA expression in individual cells was often too high to facilitate counting of individual spots, so mean fluorescence intensity (MFI) was evaluated in a zone surrounding the nucleus of each cell (Figure 2). This provided a representative sampling of RNA expression without the need for a

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secondary cell mask or segmentation of individual spots. Since monocyte-derived macrophages exhibit significant heterogeneity between individual cells, at least 5,000 cells were analyzed for each condition to assess the entire population.

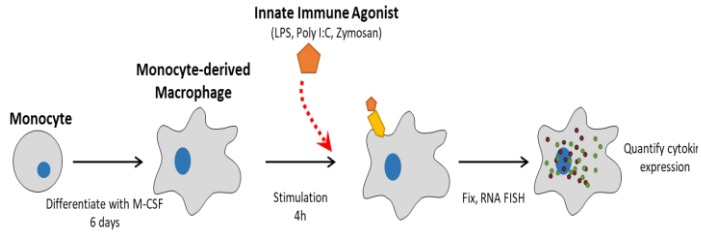

Figure 1 - Outline of the experiment.

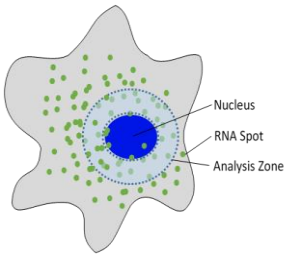

Figure 2 - Image analysis strategy. A ring-shaped zone of interest was generated around each nucleus. Mean fluorescence intensity inside each zone was determined for each RNA stain.

Results

Sufficient sample size for analysis was provided rapidly and easily as the *Hermes* system completed the scan of 1116 images in 11 minutes (captured 31 images in each of the 12 wells at 20x magnification, 3 fluorescence channels per field). MDMs expressed few copies of IL-10 and TNF mRNA in the absence of stimulation. The cytokine response differed significantly depending on the stimulus given. There was substantial diversity in the levels of expression between individual cells (Figure 3).

Quantitation of the RNA staining in each cell revealed that, while many cells expressed neither IL-10 or TNFa, a subset of cells produced large amounts of cytokine RNA (Figure 4). This varied by stimulus, with Poly I:C producing a weak TNFa response with minimal IL-10 while LPS and Zymosan induced expression of both. Zymosan induced substantially more TNFa than LPS, while LPS produced greater IL-10 induction. These differences may reflect the different pattern recognition receptors which generate responses to each ligand (TLR4 for LPS, TLR2/Dectin-1 for Zymosan and TLR3 for Poly I:C), although the expression could also be dose-dependent.

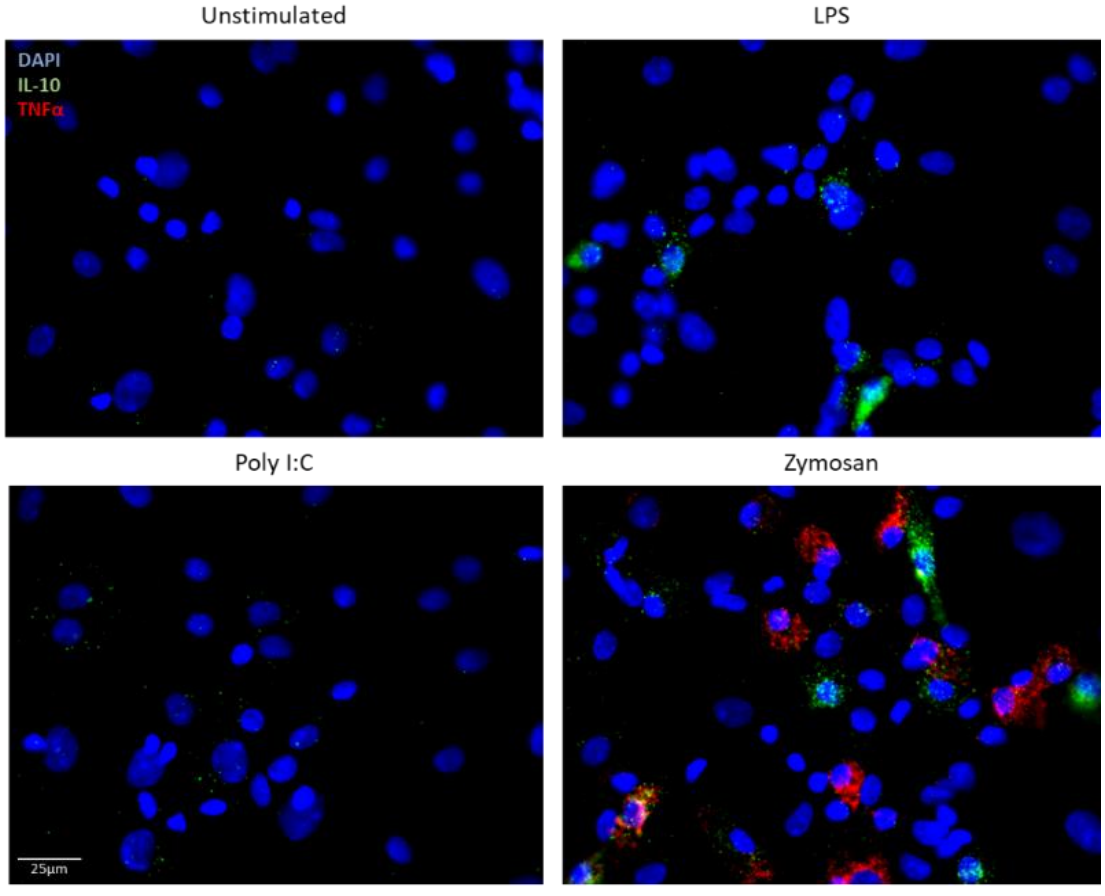

Figure 3 - Representative images from *Hermes* automated wide field microscope for RNA FISH (20x magnification) in macrophages stimulated with innate immune agonists. Nuclei were stained with DAPI (blue) while IL-10 (green) and TNFa (red) were stained by ViewRNA FISH.

It was also possible to directly compare the expression of the two cytokines within each cell (Figure 5). This revealed a bias towards expressing both TNFa and IL-10 together rather than just IL-10 alone, which may reflect IL-10's role as an immunomodulatory cytokine expressed as part of the inflammatory response.

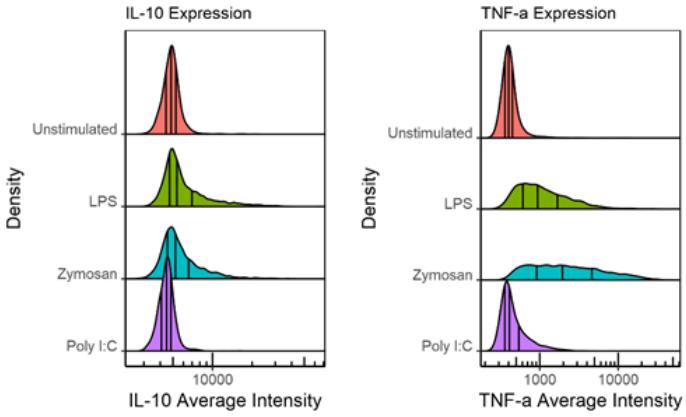

Figure 4 - Quantitation of staining for IL-10 and TNF at the single cell level. Density represents the proportion of cells with a specific MFI.

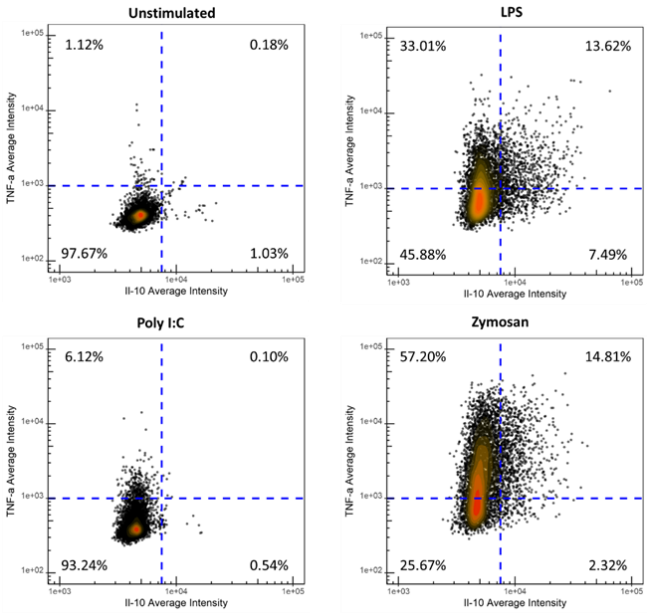

Figure 5 - Scatter/density plot of expression of IL-10 RNA against TNF RNA. Percentages of events within each segment of a plot are indicated.

Conclusion

The Hermes system was capable of high-throughput imaging of RNA FISH staining, which permitted the quantitation of cytokine expression at the single cell level. The cytokine response varied between different stimuli, with a tendency towards co-expression of IL-10 with TNF. Future work should focus on evaluating the effect of stimulus dose on the balance of inflammatory and regulatory cytokine signaling, as well as extending the assay to stain additional markers.

Data courtesy of David Stirling and Matthew Solomons (Noursadeghi Lab, Division of Infection and Immunity, University College London).

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