Application Note

Cancer Biology

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Multiplexed Detection of Cytokine Cancer Biomarkers Using Fluorescence RNA *In Situ* Hybridization and Cellular Imaging

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Abstract

Cytokines play an important role in multiple aspects of cancer, including development and advancement, treatment, and prognosis. Within the tumor environment, they contribute to tumorigenesis, tumor progression, and apoptosis. Expression of specific cytokines is also implicated in enhanced tumor cell survival rates as well as metastatic activity. While many cancer-related cytokines have been identified, the pro-inflammatory cytokines IL-6 and IL-8 are linked to a wide range of cancers including lymphoma, melanoma, breast, prostate, and colorectal cancers, among others. Specifically, increased expression of IL-6 is seen in patients with colorectal and prostate cancers.[1,2] IL-8 is also expressed in prostate cancer cells, where its presence has been linked to the metastatic potential of these cells.[3] This same role is also identified for breast cancer, where high levels of IL-8 expression increase the invasiveness of estrogen-receptor negative breast cancer cells.[4] Therefore, profiling cytokine expression can be an important diagnostic tool and predictor of cancer prognosis.

Introduction

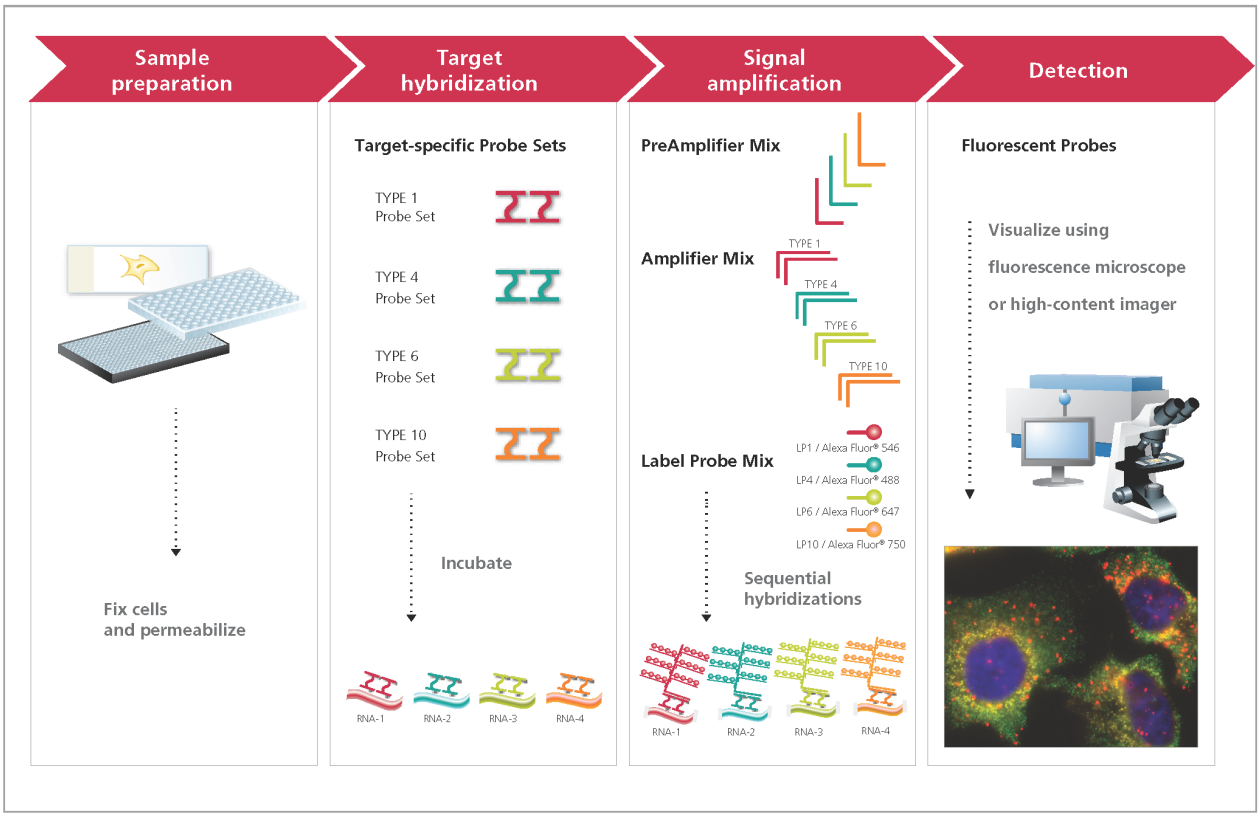
Fluorescence *in situ* hybridization (FISH) techniques are a common method to visualize nucleic acid expression at the DNA or RNA level within cells. However, the fluorescence *in situ* hybridization of localized RNA has been limited by low sensitivity, complicated workflow, and the inability to perform multiplex analysis.

This application note demonstrates a unique, nonradioactive RNA *in situ* hybridization solution that offers RNA sensitivity and multiplexed analysis for one to four mRNA targets simultaneously, in single cells, and at a single transcript detection level.

The fluorescence emanating from the amplified signal is then easily captured using a cell imaging multimode reader. With capacity for up to four fluorescence imaging cubes, detection of the multiplexed assay can be accomplished using the instrument. RNA expression levels are then determined using cellular analysis algorithms. This application note demonstrates that the combination provides an efficient, sensitive, and repeatable method to test for the presence of important predictive cancer biomarkers.

Assay principle

The ViewRNA ISH Cell Assay method (Figure 1) starts by fixing, permeabilizing, and digesting cells with protease to allow target accessibility. Then, a target-specific Probe Set hybridizes to each target mRNA, where subsequent signal amplification depends on the specific hybridization of adjacent oligonucleotide pairs. Signal amplification, using branched DNA (bDNA) technology, occurs through a series of sequential hybridization steps. PreAmplifier molecules hybridize to their respective bound oligonucleotide pair, then multiple Amplifier molecules hybridize to their respective PreAmplifier. Multiple Label Probe oligonucleotides conjugated to the fluorescent dye then hybridize to the corresponding Amplifier molecule. When fully assembled the structure has 400 binding sites for each Label Probe, and when all target-specific oligonucleotides in the Probe Set bind to the target mRNA transcript, an 8,000-fold amplification occurs for that transcript. Fluorescent signal from each amplified probe set is then detected using the Agilent BioTek Cytation 5 cell imaging multimode reader.



ViewRNA® ISH Cell Assay workflow.

Figure 1. QuantiGene ViewRNA Assay procedure from Affymetrix.

Materials and methods

Materials

Assay and experimental components

The QuantiGene ViewRNA ISH Cell Assay kit (part number QVC0001), human IL6 ViewRNA probe (part number VA1-13526), human IL8 ViewRNA probe (part number VA4-13193), and human ACTB ViewRNA probe (part number VA6-10506) were generously donated by Affymetrix (Santa Clara, CA).

U 0126 (part number 1144), and recombinant human IL-1β (part number 201-LB-005) were purchased from R&D Systems (Minneapolis, MN).

Cells

HCT116 colorectal carcinoma cells (part number CCL-247), MDA-MB-231 breast adenocarcinoma cells (part number HTB-26), and DU 145 prostate carcinoma cells (part number HTB-81) were purchased from ATCC (Manassas, VA).

Agilent BioTek Cytation 5 cell imaging multimode reader

Agilent BioTek Cytation 5 is a modular multimode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield, and phase contrast. The instrument can perform fluorescence imaging in up to four channels. With special emphasis on live cell assays, the Cytation 5 cell imaging multimode reader features temperature control to 65 °C, CO_2/O_2 gas control, and dual injectors for kinetic assays. Integrated Agilent BioTek Gen5 microplate reader and imager software controls Cytation 5. The instrument was used to perform fluorescence imaging of the ViewRNA assay using DAPI, GFP, RFP, and Cy5 imaging channels with 20x or 40x objectives, in addition to image and cellular analysis.

Methods

Cell plating

HCT116, MDA-MB-231, and DU 145 cells were added to a poly-L-lysine coated 96-well imaging plate, at a concentration of 2.0×10^4 cells/well and incubated overnight at 37 °C/5% CO_2. Negative control wells were then serum starved for 18 hours at 37 °C/5% CO_2 by replacing complete media with media containing 0.1% serum.

Assay procedure

Per the stimulation experiments, after culture media was removed from the wells, IL-1β was added to DU 145 cells at concentrations ranging from 2 to 0 ng/mL, the plate was covered and incubated for 3 hours at 40 °C. Per the inhibitor experiments, after culture media was removed from the wells, U 0126 kinase inhibitor was added to DU 145 and MDA-MB-231 cells at concentrations ranging from 10 to 0 µM for 30 minutes. DU 145 cells were then stimulated with IL-1β as previously described. After incubation, the assay procedure proceeded according to the Affymetrix User Manual: QuantiGene ViewRNA ISH Cell Assay user manual (18801, Rev. A 110525) with modifications based on the Affymetrix technical note: Guidelines and Procedure Modifications for Running QuantiGene ViewRNA ISH Cell Assay in a 96-Well Plate Format (Rev. A 110701), including reagent volumes of 60 µL/well and wash step volumes of 150 µL/well. Other modifications included optimizing the wash step iterations from three to one due to the loosely adherent cells.

Results and discussion

Fluorescently labeled mRNA imaging and analysis

The ability to accurately image fluorescently labeled mRNA molecules expressed in cancer cell lines was proven using HCT116 and MDA-MB-231 cells. Positive control cells were maintained in complete medium, while negative control cells were serum starved for 18 hours to lower cytokine expression. ViewRNA probes were added to positively label IL-6, IL-8, and ACTB mRNA, in addition to a DAPI nuclear probe. RFP, GFP, Cy5, and DAPI fluorescent imaging channels, respectively, were used to image the probes following completion of the ISH cell assay procedure. As shown in Figure 2, fluorescent signals from each probe were accurately identified using optimized exposure settings, 20x or 40x objectives, and the previously listed Cytation 5 imaging channels.

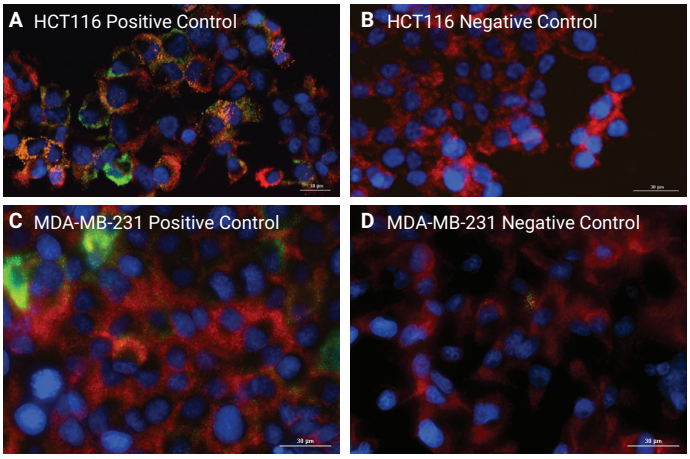


Figure 2. Positive and negative control well imaging. Zoomed 20x images of HTC116 cells as (A) positive control and (B) negative control. 40x images of MDA-MB-231 cells as (C) positive control and (D) negative control. Blue: DAPI stained nuclei; Green: labeled IL-8 mRNA probe; Orange: labeled IL-6 mRNA probe; Red: labeled ACTB mRNA probe.

To quantify cytokine expression, cellular analysis was first carried out with the Cytation 5 DAPI channel to determine the number of cells per well (Figure 3A). Image analysis was then performed with the appropriate channel to measure the fluorescent signal from each labeled cytokine probe set (IL-6 or IL-8) above background per image (Figure 3B). The ratio of fluorescent signal per cell was then used to assess cytokine expression for each test condition (Figure 4).

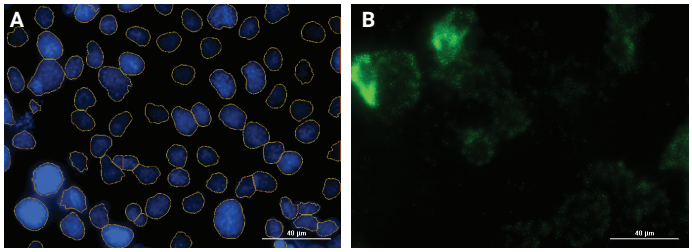


Figure 3. Fluorescent signal per cell analysis. (A) Object masks placed around DAPI labeled nuclei using Agilent BioTek Gen5 cellular analysis; (B) Image analysis of fluorescent labeled IL-8 signal.

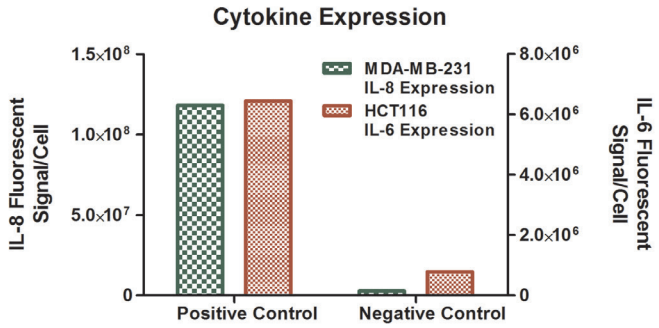


Figure 4. Fluorescent signal per cell from IL-8 expression in MDA-MB-231 cells and IL-6 expression in HCT116 cells.

The images in Figure 2 and fluorescent signal per cell values in Figure 4 demonstrated that IL-6 and IL-8 mRNA expression from positive control cells were accurately quantified using the ViewRNA ISH cell assay and Cytation 5. Furthermore, changes in cytokine expression were also identified as witnessed by the signal reduction per cell from serum starved cells.

Induction of cytokine mRNA expression

Following the assay procedure, the known stimulator IL-1β was added to DU 145 cells in a dose-dependent manner to measure induction of cytokine mRNA expression. While an increase in mRNA was seen with IL-6 and IL-8 cytokines (Figure 5), IL-8 expression was more sensitive to IL-1β stimulation (Figure 6), which agreed with previously published reports.[5] This validated the ability of the assay and assessment method to yield accurate results. The reduction in expression of both cytokines at the highest dose of IL-1β is attributable to cytotoxicity.

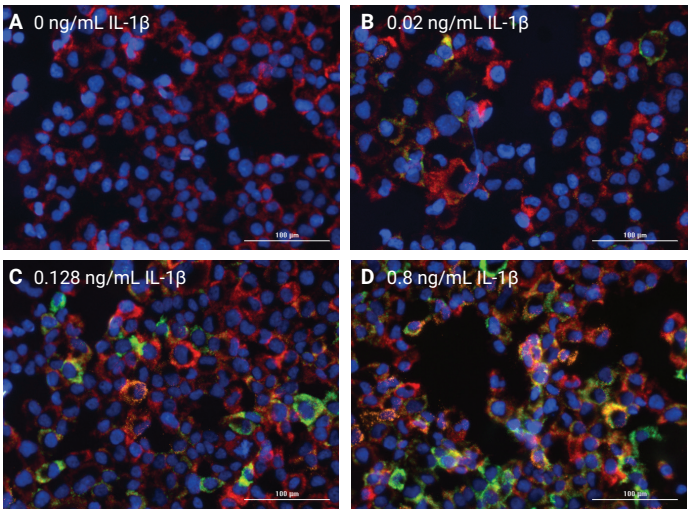


Figure 5. IL-1β treated DU 145 cells. 20x overlaid images showing IL-6, IL-8, and ACTB fluorescent mRNA probe signal and DAPI stained nuclei following three-hour incubation with (A) 0; (B) 0.02; (C) 0.128; or (D) 0.8 ng/mL IL-1β. Blue: DAPI stained nuclei; Green: labeled IL-8 mRNA probe; Orange: labeled IL-6 mRNA probe; Red: labeled ACTB mRNA probe.

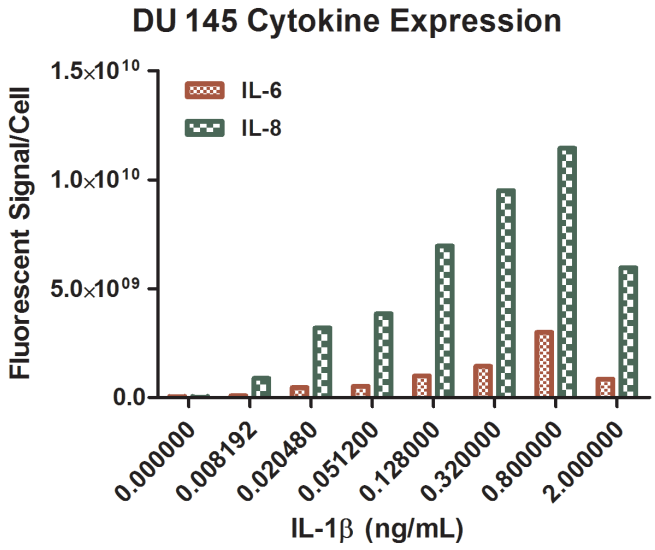


Figure 6. IL-6 and IL-8 mRNA expression in DU 145 cells following IL-1β stimulation.

Inhibition of cytokine mRNA expression

Independent research has implicated the mitogen-activated protein kinase (MAPK) in regulation of IL-8, and demonstrated that treatment with the MAPK/ERK inhibitor U 0126 reduces expression of the inflammatory cytokine in DU 145 and MDA-MB-231 cells.[5,6] To confirm this phenomenon and validate the ability of the assay and analysis process to monitor cytokine inhibition, varying concentrations of U 0126 were added to each cell type and incubated for 30 minutes. DU 145 cells were then stimulated with 1 ng/mL IL-1β for three hours, while MDA-MB-231 cells remained unstimulated. Cell counts and image analysis using the GFP and RFP imaging channels were completed on all test wells following incubation to assess IL-8 and IL-6 cytokine mRNA expression, respectively, following U 0126 treatment.

Images (Figure 7) and calculated fluorescent signal per cell values (Figure 8) confirmed the effect that U 0126 has on mRNA expression of the IL-8 inflammatory cytokine. Furthermore, the results validated the sensitivity of the ViewRNA ISH cell assay and image-based analysis carried out by the Cytation 5 to accurately identify changes in mRNA expression following treatment with inhibitory molecules.

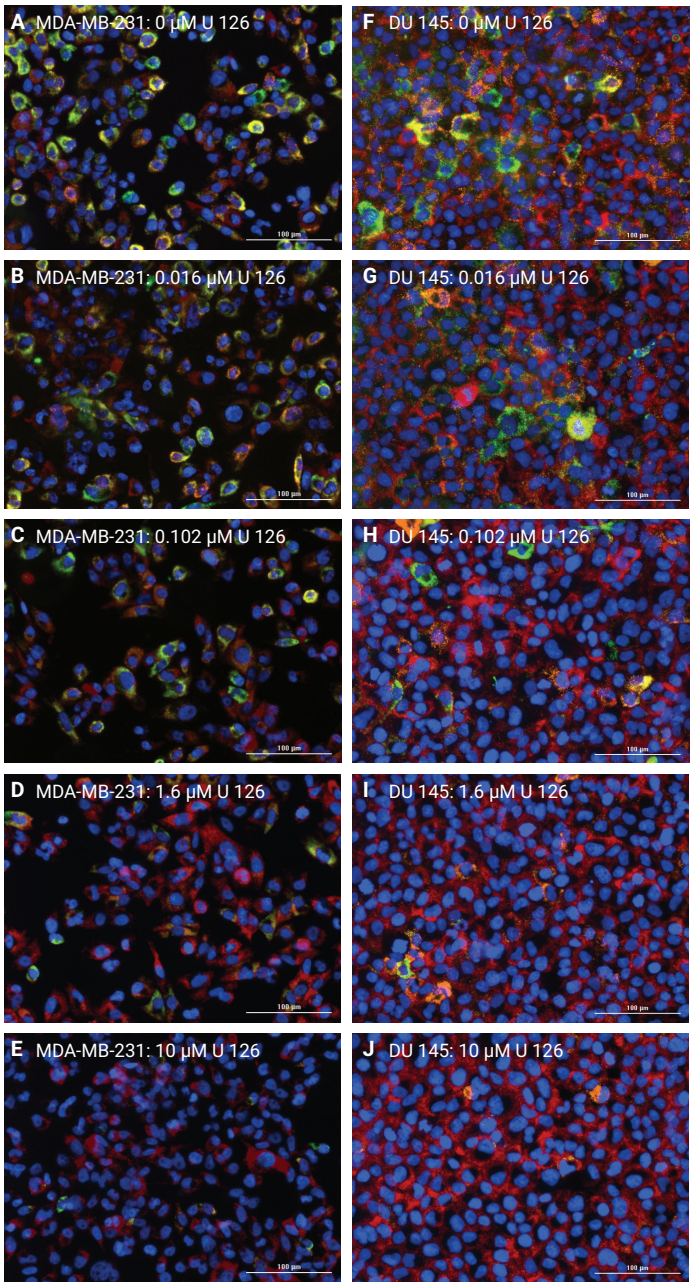


Figure 7. U 0126 inhibition of IL-8 mRNA expression. 20x overlaid images showing IL-6, IL-8, and ACTB fluorescent mRNA probe signal and DAPI stained nuclei following U 0126 treatment of (A-E) MDA-MB-231; or (F-J) DU 145 cells. Blue: DAPI stained nuclei; Green: labeled IL-8 mRNA probe; Orange: labeled IL-6 mRNA probe; Red: labeled ACTB mRNA probe.

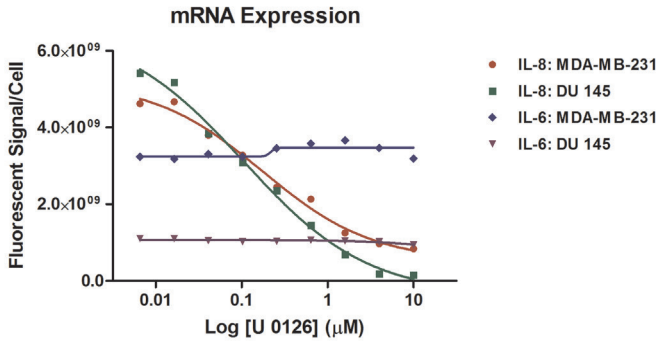


Figure 8. IL-8 and IL-6 mRNA expression in MDA-MB-231 and DU 145 cells following U 0126 treatment.

Conclusion

The ViewRNA ISH cell assay kit and probes from Affymetrix provide a sensitive method to detect basal, as well as subtle changes in mRNA expression, following treatment with stimulatory or inhibitory molecules. Also, the specificity of each ViewRNA probe, and the multichannel fluorescent imaging capabilities of Agilent BioTek Cytation 5 cell imaging multimode reader allow for simultaneous imaging and analysis of multiple probes on a single imaging procedure. The combination of fluorescent imaging and cellular/image analysis inherent to Cytation 5 with Agilent BioTek Gen5 microplate reader and imager software, provides a robust method to accurately calculate the fluorescent signal from each probe per the number of cells in each image. Finally, the combination of detection, imaging and analysis provide a sensitive, flexible and high-throughput method to detect mRNA expression of important cancer-related cytokine biomarkers.

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