

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 has also been 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 have been linked to a wide range of cancers including lymphoma, melanoma, breast, prostate, and colorectal cancers, among others. Specifically, increased expression of IL-6 has been seen in patients with colorectal (Komoda, *et al.*, 1998) and prostate (Culig, *et al.*, 2012) cancers. IL-8 is also expressed in prostate cancer cells, where its presence has been linked to the metastatic potential of these cells (Aalinkeel, *et al.*, 2004). This same role was also identified for breast cancer, where high levels of IL-8 expression increase the invasiveness estrogen-receptor negative breast cancer cells (Freund, *et al.*, 2003). Therefore profiling cytokine expression can be an important method as a diagnostic tool and predictor of cancer prognosis.

Fluorescence based *in situ* hybridization (FISH) techniques have become a common method to visualize nucleic acid expression at the DNA or RNA level within cells. However the fluorescence *in situ* hybridization of RNA has always been limited by low sensitivity, complicated workflow and the inability to perform multiplex analysis. Here we describe a unique, non-radioactive RNA *in situ* hybridization solution that offers single-molecule RNA sensitivity and multiplexed analysis for one to four RNA targets. The fluorescence emanating from the amplified signal associated with each mRNA molecule can be easily captured using a novel cell imaging multi-mode reader. With up to four fluorescence imaging cubes capable of being installed in the instrument, simultaneous detection of the multiplexed assay can be accomplished. Levels of RNA expression are then determined using cellular analysis algorithms to identify the number of mRNA molecules per cell in each image. The combination provides an efficient, sensitive and repeatable method to test for the presence of important predictive cancer biomarkers.

BioTek Instrumentation

Cytation™ 5 Cell Imaging Multi-Mode Reader. Cytation 5 is a modular multi-mode 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 in a single step. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO₂/O₂ gas control and dual injectors for kinetic assays. Integrated Gen5™ Data Analysis Software controls Cytation 5. The instrument was used to perform fluorescence imaging of the ViewRNA assay using DAPI, GFP, RFP, and Cy5 imaging channels and 20x or 40x objectives, in addition to image and cellular analysis.

QuantiGene® ViewRNA ISH Cell Assay

In situ hybridization techniques are used to visualize DNA or localized RNAs within cells. The QuantiGene® ViewRNA ISH Cell Assay from Affymetrix incorporates an *in situ* hybridization technology that has the sensitivity and robustness to visualize up to 4 target mRNAs simultaneously, in single cells, at a single transcript detection level.

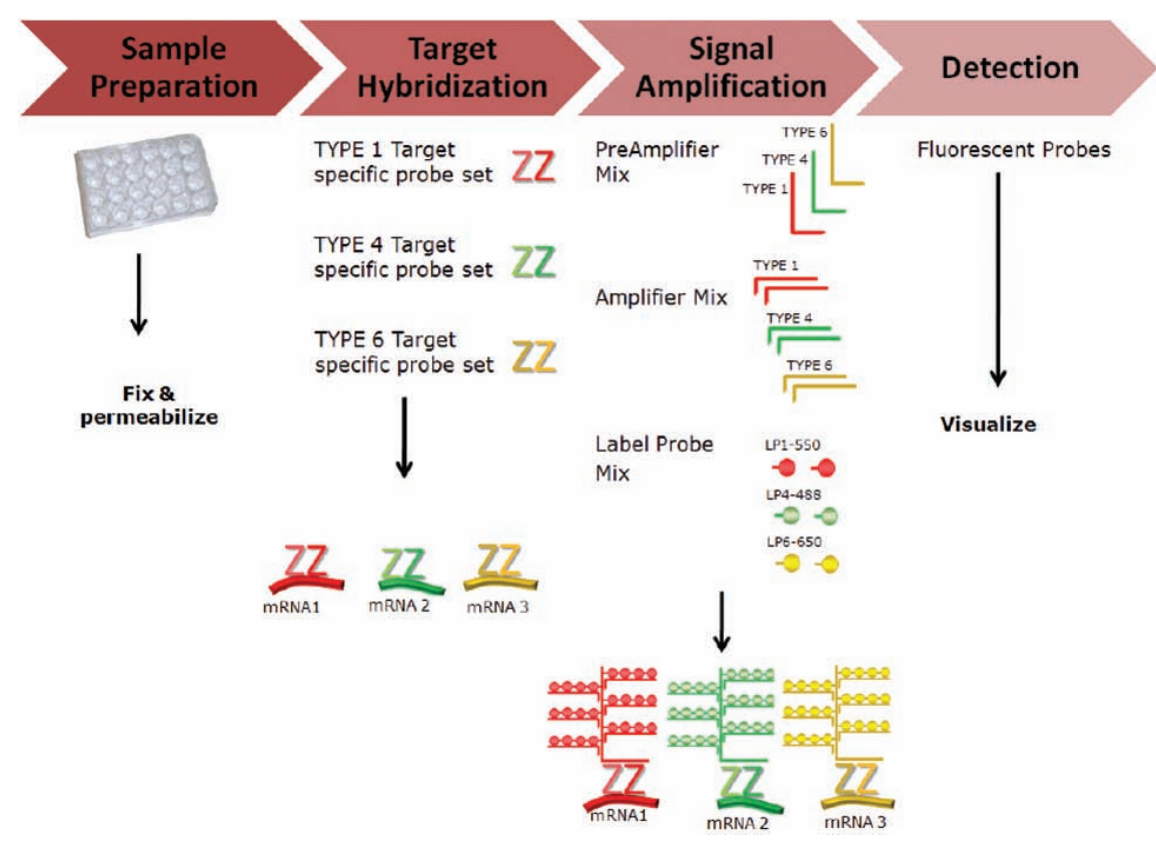


Figure 1. ViewRNA Assay Procedure.

Sample Preparation. Cells are fixed, permeabilized and digested by protease to allow target accessibility. **Target Hybridization.** A target-specific Probe Set hybridizes to each target mRNA. Subsequent signal amplification is predicated on specific hybridization of adjacent pairs of oligonucleotides. **Signal Amplification.** Signal amplification, using bDNA technology, is achieved via a series of sequential hybridization steps. The PreAmplifier molecules hybridize to their respective pair of bound Probe Set oligonucleotides, then multiple Amplifier molecules hybridize to their respective PreAmplifier. Next, multiple Label Probe oligonucleotides conjugated to the fluorescent dye hybridize to the corresponding Amplifier molecule. A fully assembled signal amplification “tree” has 400 binding sites for each Label Probe. When all target-specific oligos in the Probe Set bind to the target mRNA transcript, an 8,000 fold amplification occurs for that one transcript. **Detection.** Target mRNAs are visualized using a standard fluorescent microscope.

Methods and Methods

Assay and Experimental Components: The QuantiGene ViewRNA ISH Cell Assay Kit (Catalog No. QVC0001), Human IL6 ViewRNA® probe (Catalog No. VA1-13526), Human ViewRNA probe (Catalog No. VA4-13193), and Human ACTB ViewRNA probe (Catalog No. VA6-10506) were generously donated by Affymetrix (Santa Clara, CA).

U 0126 (Catalog No. 1144), and recombinant human IL-1β (Catalog No. 201-LB-005) were purchased from R&D Systems (Minneapolis, MN).

Cells: HCT116 colorectal carcinoma cells (Catalog No. CCL-247), MDA-MB-231 breast adenocarcinoma cells (Catalog No. HTB-26), and DU 145 prostate carcinoma cells (Catalog No. HTB-81) were purchased from ATCC (Manassas, VA).

Assay Procedure: **Cell Plating:** HCT116, MDA-MB-231, and DU 145 cells were added to a 96-well imaging plate, previously coated with poly-L-lysine, at a concentration of 2.0x10⁴ cells/well and incubated overnight at 37 °C/5% CO₂. Negative control wells were then serum starved for 18 hours at 37 °C/5% CO₂ by replacing complete media with media containing 0.1% serum. **Stimulation Experiments:** IL-1β was added to DU 145 cells at concentrations ranging from 2-0 ng/mL and incubated for 3 hours. **Inhibitor Experiments:** U 0126 kinase inhibitor was added to DU 145 and MDA-MB-231 cells at concentrations ranging from 10-0 μM for 30 minutes. DU 145 cells were then stimulated with IL-1β as previously described. **Assay Performance and Imaging:** The ViewRNA ISH cell assay was then performed as previously described.

Fluorescently Labeled mRNA Imaging and Analysis

The ability to accurately image fluorescently labeled mRNA molecules expressed in cancer cell lines was first performed 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.

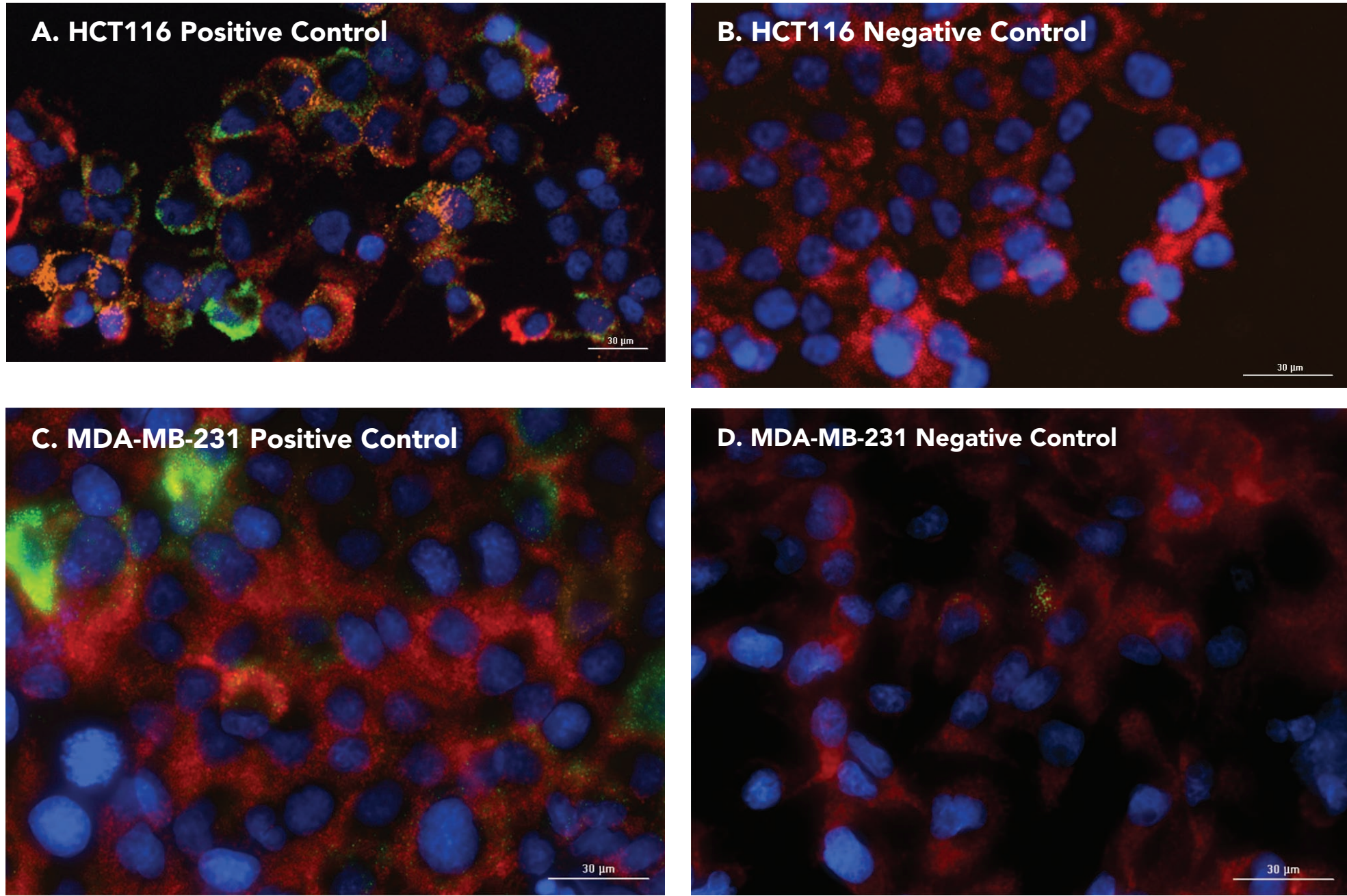


Figure 2. Positive and Negative Control Well Imaging. (A) Zoomed in 20x image of positive control HCT116 cells; (B) Zoomed in 20x image of negative control HCT116 cells; (C) 40x image of positive control MDA-MB-231 cells; (D) 40x image of negative control MDA-MB-231 cells. Blue: DAPI stained nuclei; Green: labeled IL-8 mRNA probe; Orange: labeled IL-6 mRNA probe; Red: labeled ACTB mRNA probe.

The images in Figure 2 illustrate that fluorescent signals from each probe were able to be accurately identified using optimized exposure settings, 20x or 40x objectives, and the previously listed imaging channels of the Cytation 5.

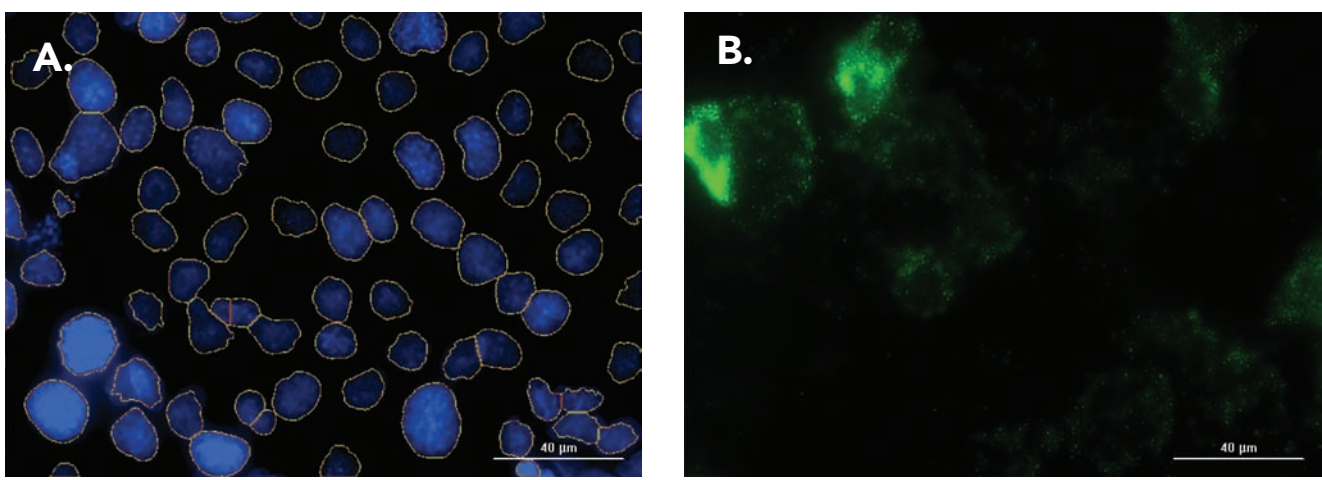


Figure 3. Fluorescent signal per cell analysis using Cytation 5 and Gen5 Data Analysis Software. (A) Object masks placed around DAPI labeled nuclei using Gen5 cellular analysis; (B) Image analysis of fluorescent labeled IL-8 signal.

Quantification of cytokine expression per image was then accomplished in the following manner. Cellular analysis was first carried out with the 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.

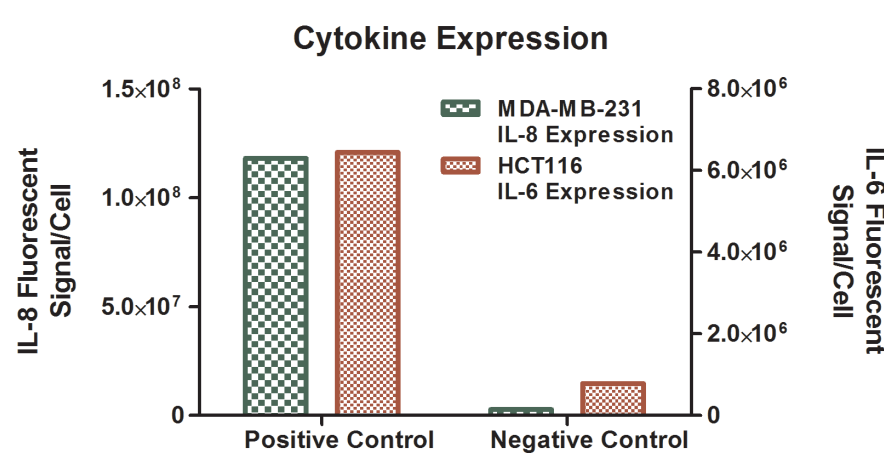


Figure 4. Fluorescent signal per cell from IL-8 expression in MDA-MB-231 cells and IL-6 expression in HCT116 cells.

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

Induction of Cytokine mRNA Expression

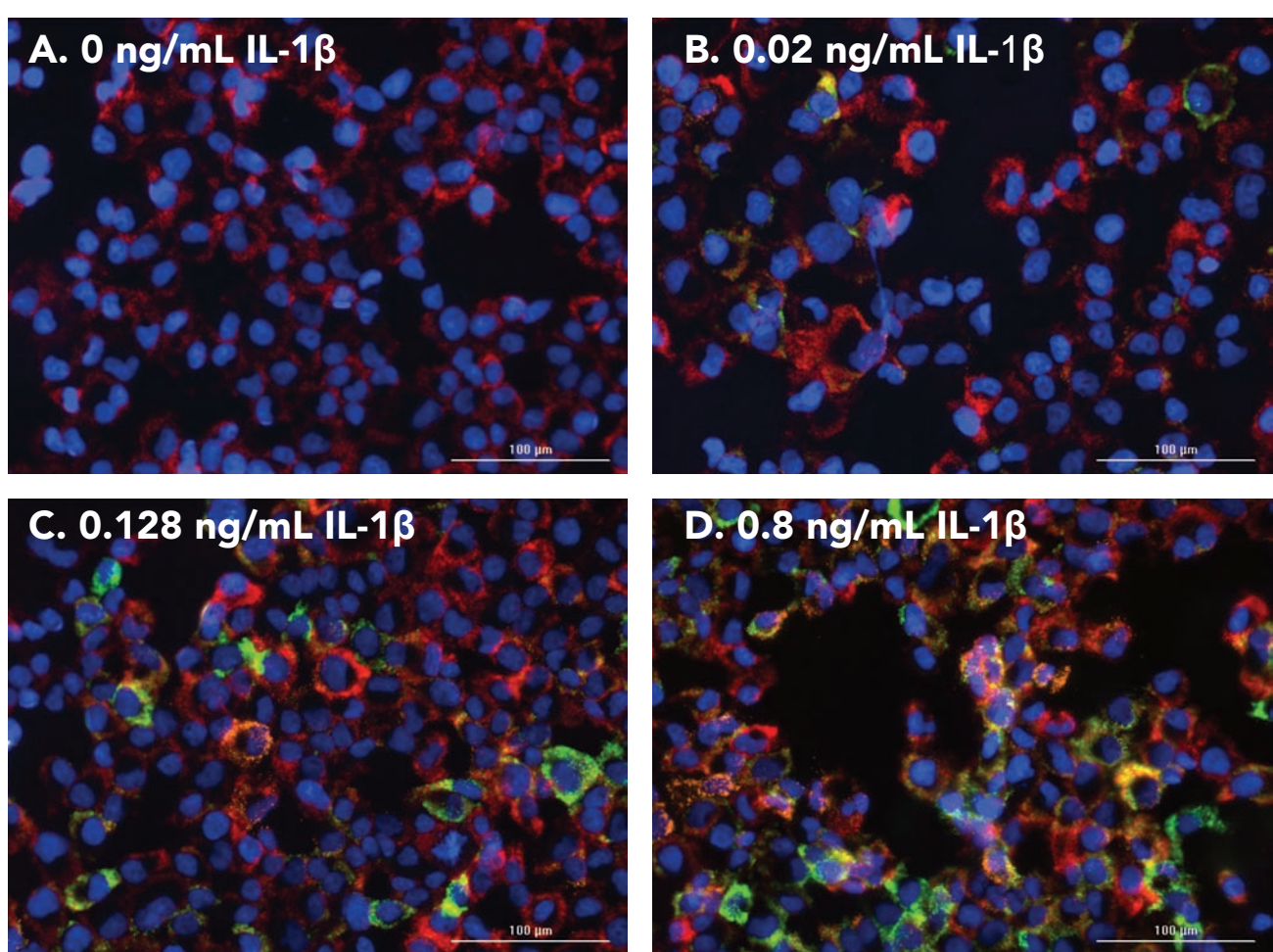


Figure 5. IL-1β treated DU 145 cells. 20x overlaid images showing IL-6, IL-8, and ACTB fluorescent mRNA probe signal and DAPI stained nuclei following three hour incubation with (A) 0; (B) 0.02; (C) 0.128; or (D) 0.8 ng/mL IL-1β. Blue: DAPI stained nuclei; Green: labeled IL-8 mRNA probe; Orange: labeled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

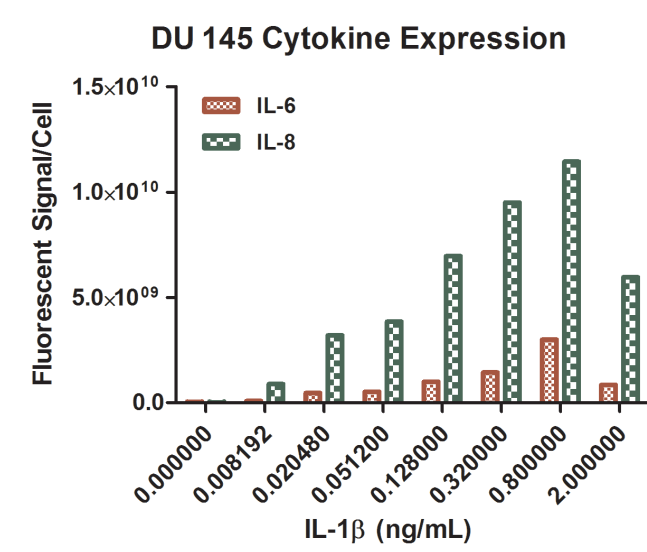


Figure 6. IL-6 and IL-8 mRNA expression in DU 145 cells following IL-1β stimulation.

Dose dependent stimulation of mRNA expression for both IL-6 and IL-8 was seen following IL-1β incubation. While an increase in mRNA is seen with both cytokines, IL-8 expression is more sensitive to IL-1β stimulation, agreeing with the findings of Kooijman, *et al.*; further validating the ability of assay and assessment method to yield accurate results.

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 (Kooijman, *et al.*) and MDA-MB-231 (Chelouche-Lev, *et al.*, 2004) cells. 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. Following incubation assay and imaging were once again performed on all test wells.

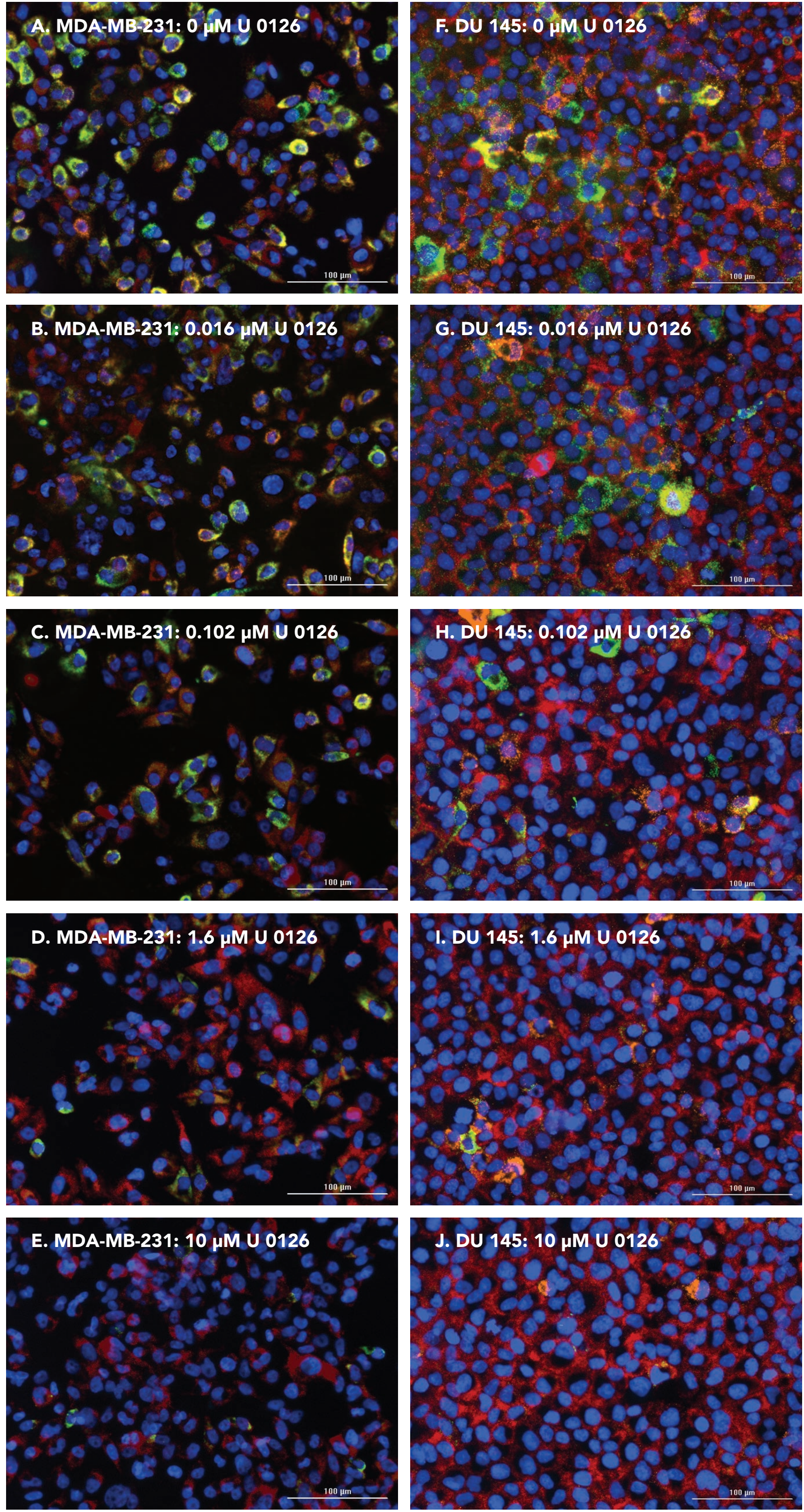


Figure 7. U 0126 inhibition of IL-8 mRNA expression. 20x overlaid images showing IL-6, IL-8, and ACTB fluorescent mRNA probe signal and DAPI stained nuclei following U 0126 treatment of (A-E) MDA-MB-231; or (F-J) DU 145 cells. Blue: DAPI stained nuclei; Green: labeled IL-8 mRNA probe; Orange: labeled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

Cell counts and image analysis using the GFP imaging channel were completed to assess IL-8 cytokine mRNA expression following U 0126 treatment.

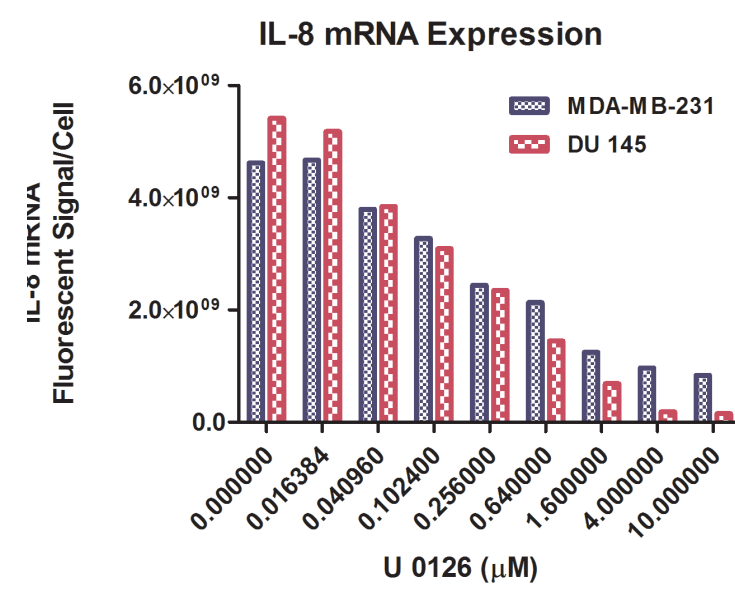


Figure 8. IL-8 mRNA expression in MDA-MB-231 and DU 145 cells following U 0126 treatment.

The images in Figure 7 and calculated fluorescent signal per cell values reported in Figure 8 confirm the effect that U 0126 has on mRNA expression of the IL-8 inflammatory cytokine previously reported in the literature. The results further validate 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.

Conclusions

1. 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.
2. The combined fluorescent imaging, in addition to cellular and image analysis capabilities of the Cytation 5 and Gen5 Data Analysis Software, supply a robust method to accurately calculate the fluorescent signal from each probe per the number of cells in each image.
3. The specificity of each ViewRNA probe and multichannel fluorescent imaging capabilities of the Cytation 5 allow for simultaneous imaging and analysis of multiple probes in a single imaging procedure.
4. The combination of detection method, and imaging and analysis capabilities, provide a sensitive, flexible, and high-throughput method to detect mRNA expression of important cancer related cytokine biomarkers.