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 are thought to have 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., 1990) and prostate (Curigli, et al., 2012) cancer. IL-8 is also expressed in prostate cancer cells, where its presence has been linked to the metastatic phenotype (Pereira, et al., 2011). This study was also identified as breast cancer, where high levels of IL-8 expression increase the invasive estrogen receptor-negative breast cancer cells (Finland, et al., 2009). Therefore profiling cytokine expression can be an important method as a diagnostic tool and predictor of cancer prognosis.

Fluorescence 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 multiple analyses. Here we describe a novel, non-radioactive RNA in situ hybridization solution that offers single-molecule RNA sensitivity and multiplexed analyses 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 capac parallels 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. This method provides an effective, sensitive and rapid method to test for the presence of important predictive cancer biomarkers.

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 is 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. Special emphasis on live-cell assays, Cytation 5 features temperature control to 60 °C, CO2 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 RNAs simultaneously, in single cells, at a single transcript detection level.

Methods and Methods

Assay and Experimental Components: The QuantiGene ViewRNA ISH Cell Assay Kit (Catalog No: QVC001), Human IL-6 ViewRNA® probe (Catalog No. VA1-13526), Human IL-8 ViewRNA probe (Catalog No. VA44-13179), and Human ACTB ViewRNA probe (Catalog No. VA44-13065) were generously donated by Affymetrix (Santa Clara, CA).

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).

U 0126 (Catalog No. 104-0116) and recombinant human IL-1β (Catalog No. 201-LB-005) were purchased from R&D Systems (Minneapolis, MN).

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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 stimulation. ViewRNA probes were added to positively label IL-6, IL-8, and ACTB mRNA, in addition to a GAPDH nuclear probe. RFP, GFP, Cy5, and FITC fluorescent imaging channels, respectively, were used to image the probes following completion of the ISH cell assay procedure.

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 inhibitor U 0126 reduces expression of the inflammatory cytokine in DU 145 (Kosimono, et al.) and MDA-MB-231 (Drouilhet-Levaux, et al., 2008) 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 test cell line and incubated for 30 minutes. DU 145 cells were then stimulated with 1 ng/mL IL-8 for three hours, while MDA-MB-231 cells remained unstimulated. Following incubation assay and imaging were once again performed on all test wells.

Induction of Cytokine mRNA Expression

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.

Results and Discussion

Multiplexed detection of cytokine cancer biomarkers was accomplished. Levels of IL-6 and IL-8 expression were then determined using cellular analysis algorithms to identify the number of mRNA molecules per cell in each image. This method provides an effective, sensitive and rapid method to test for the presence of important predictive cancer biomarkers.

**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 are thought to have 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., 1990) and prostate (Curigli, et al., 2012) cancer. IL-8 is also expressed in prostate cancer cells, where its presence has been linked to the metastatic phenotype (Pereira, et al., 2011). This study was also identified as breast cancer, where high levels of IL-8 expression increase the invasive estrogen receptor-negative breast cancer cells (Finland, et al., 2009). Therefore profiling cytokine expression can be an important method as a diagnostic tool and predictor of cancer prognosis.

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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 parts of oligonucleotides. Signal Amplification. Signal amplification, using ISHNA technology, is achieved via a series of sequential hybridization reactions in which Pre Amplifier molecules hybridize to their respective Probe Set oligonucleotides, then multiple Amplifier molecules hybridize to their respective Pre Amplifier. 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 mRNA probes are visualized using a standard fluorescent microscope.

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.

Image analysis of fluorescent nuclei using Gen5 cellular analysis; Data Analysis Software. (A) Object mask used around DAPI-labeled nuclei using Gen5 cellular analysis; (B) Image analysis of fluorescently labeled IL-6 signal.