Fluorescent In Situ Hybridization In Suspension Analysis Using ImageStream® Multispectral Imaging Flow Cytometry

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Abstract

Amnis Corporation has developed the ImageStream 100 multispectral imaging flow cytometer. The platform combines the morphological capabilities of multiple forms of microscopy with the sample handling and quantitative power of flow cytometry. The high throughput capabilities of the ImageStream instrument are well suited for chromosomal Fluorescent In Situ Hybridization (FISH) analysis of large numbers of cells. Amnis' IDEAS™ software package allows quantitative, objective spot counting and analysis. In order to convert from a slide format to a flow format Amnis has developed FISH In Suspension (FISH-IS™) protocols. Applications include the detection of aneuploidy in sperm and other cell types, detection of gene amplification and translocation associated with cancer, and detection of residual disease after bone marrow transplantation. Presented is the high throughput quantitation of aneuploidy in human sperm using probes for chromosomes 8 and Y.

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Introduction

In recent years, the technique of *in situ* hybridization has contributed greatly to progress in molecular cytogenetics. This technique has allowed the linkage of DNA sequence with chromosomal location and gene expression at the tissue and cellular level.

DNA:DNA *in situ* hybridization has evolved into a competent tool for examining gene location, rearrangement and amplification. It has been used to determine evolutionary relationships of sequence data with physical mapping to chromosomal regions and DNA abnormalities associated with disease states. In addition, DNA:DNA *in situ* hybridization is used to detect germ cell chromosomal abnormalities as a predictor of birth defect risk.

Techniques for performing *in situ* hybridization are advancing as well. For instance, radioactively-labeled probes have largely been replaced by probes labeled with fluorochromes (fluorescent *in situ* hybridization, FISH), which has in turn allowed the development of multiplexing techniques.

To a large extent, FISH is performed on cells adhered to microscope slides. Data collection is microscope-based and generally performed manually, although automated microscopes with digital image collection are becoming more commonplace.

Flow cytometry's high speed and fluorescence sensitivity make it an attractive technique for FISH quantitation, but the lack of fluorescence signal localization has hindered its use except in specialized applications like aggregate telomere length measurements.

Amnis has developed the ImageStream 100 flow imaging platform that simultaneously captures brightfield, darkfield, and four channels of fluorescence imagery of cells in flow with high sensitivity and at speeds of up to 100 cells per second. The acquisition of imagery as well as fluorescence intensity data makes the IS100 platform ideally suited for data collection for cells hybridized with fluorochrome labeled nucleic acid probes.

Here the technique for hybridizing somatic (T cell lymphoma) and germ cells (sperm) with a labeled nucleic acid probe is described and preliminary data collected by the IS100 and analyzed are shown.

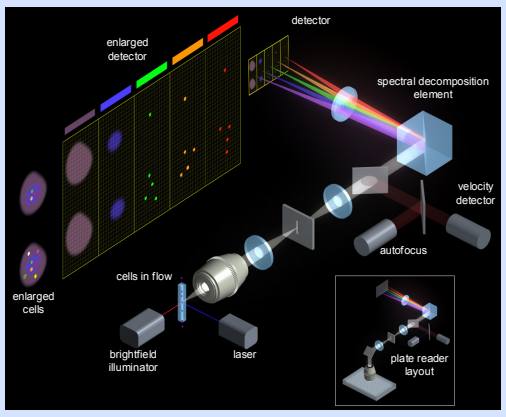

Figure 1: The ImageStream Architecture

ImageStream is a novel technology designed to image rapidly-moving objects in flow or on substrates with high sensitivity, high image fidelity, and in multiple simultaneous imaging modes. As shown in the Figure above, cells are hydrodynamically focused within a flow cuvette and are illuminated from the side and from behind with lasers or other light sources. Fluorescence, side scatter, and transmitted light from cells is imaged by an objective lens and relayed to a spectral decomposition element, which divides the imagery into spectral bands located side-by-side across the detector. Different spectral bands are used for different imaging modes or different colors of fluorescence imagery. For example, laser side scatter produces a darkfield image in the laser's spectral band while transmitted red light produces a brightfield image in the red spectral band. Because all the channels are in spatial register, image analysis is greatly facilitated and the imagery can be readily reconstructed for visual interpretation after quantitative analysis. High sensitivity is achieved by operating the CCD in Time Delay Integration (TDI) mode. TDI imaging is a method of electronically panning the detector to track object motion. TDI operation results in signal collection times that can exceed ten milliseconds, orders of magnitude longer than conventional flow cytometry, while preserving image fidelity and throughput.

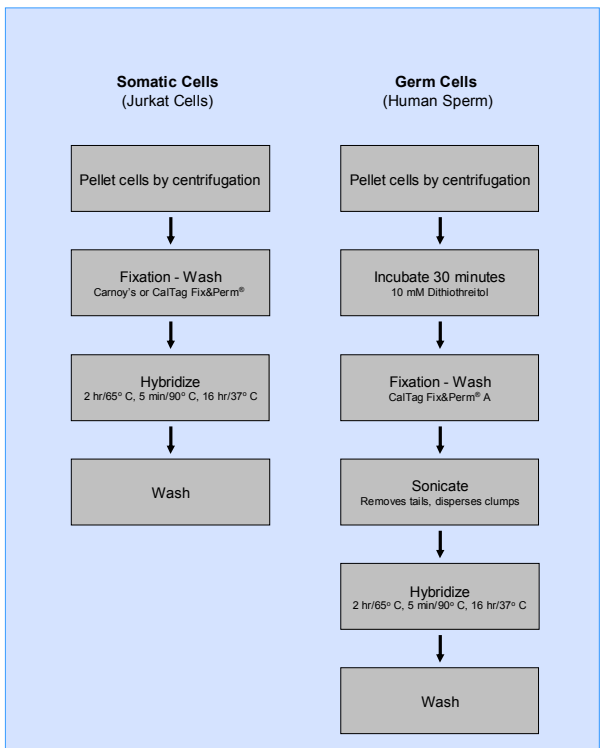

Figure 2: Fluorescent In Situ Hybridization in Suspension (FISH-IS) Protocol in Somatic and Germ Cells

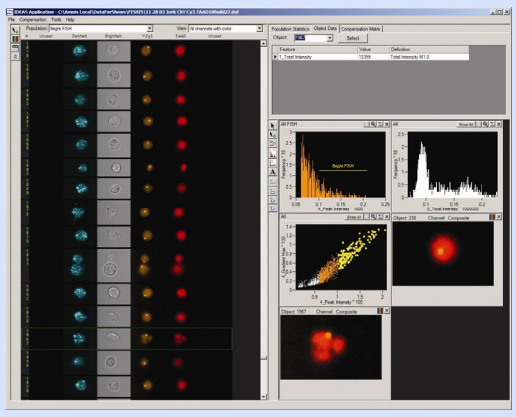

Figure 3: Multispectral Flow Imagery of Jurkat Cells Hybridized with a Y Chromosome Probe

Jurkat cells were subjected to FISH-IS with a Cy3®-labeled Chromosome Y probe according to the protocol of Figure 2 and then stained with the DNA binding dye 7-AAD to identify the nucleus. Darkfield (laser side scatter), brightfield (transmitted light), Cy3® fluorescence, and 7-AAD fluorescence imagery from several thousand cells were acquired on the ImageStream100 imaging flow cytometer at approximately 100 cells per second. Data were then analyzed using Amnis' IDEAS™ analysis program (shown above).

The left side of the IDEAS interface is the Image Gallery, used to display thumbnail images of each cell. In the display format shown above, each cell's serial number, darkfield (pseudocolor blue), brightfield, Cy3® (pseudocolor yellow) and 7-AAD (pseudocolor red) images are shown left to right in a single row. The right side of the interface is the Workspace, used to plot any of the up to 200 parameters that are automatically calculated from each cell's imagery. Dots in bivariate plots are linked to the corresponding cell imagery, which can be visualized with a mouse click to help define gating boundaries. Once a gate is defined, that population's cell imagery can be inspected in the Image Gallery (a "virtual cell sort".) The upper left plot in the workspace is a histogram of the Cy3® peak intensity. Cells with high peak intensities were gated into a population labeled "Bright FISH" and displayed in the Image Gallery.

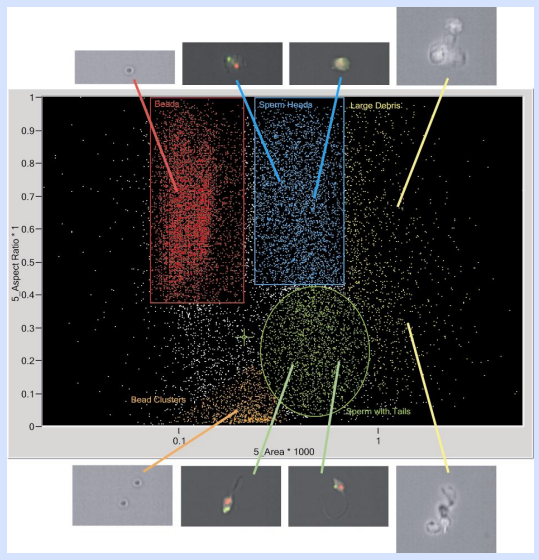

Figure 4: Segregation of Object Populations Using Brightfield Imagery

Human sperm cells were subjected to the FISH-IS protocol of Figure 2 using a Cy3® labeled Chromosome Y probe and an Alexa Fluor® 488 labeled Chromosome 8 probe. Prior to analysis for FISH spot content, the image data were segregated into multiple populations using the automatically calculated area and aspect ratio of the brightfield imagery. Several populations are evident, including: polystyrene beads (red gate), bead clusters (yellow), sperm heads (blue gate), sperm with tails (green gate) and aggregated cells and cellular debris. The 3 micron diameter polystyrene beads were spiked into the sperm sample prior to imaging in order to facilitate autofocus and TDI synchronization. The "Sperm Heads" and "Sperm with Tails" populations were further analyzed to identify cells with well-defined FISH spots below.

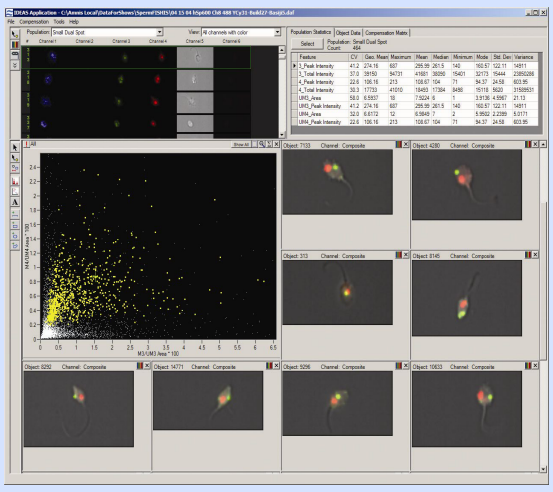

Figure 5: FISH Spot Analysis in Human Sperm

Human sperm cells were subjected to the FISH-IS protocol of Figure 2 using a Cy3® labeled Chromosome Y probe (pseudocolored red) and an Alexa Fluor® 488 labeled Chromosome 8 probe (pseudocolored green). Images of both probes, along with darkfield (pseudocolored blue) and brightfield were acquired from over 15,000 cells. Cells with well-focused FISH spots were identified by defining two masks for each fluorescent image: Mask 1 was set at a high normalized intensity threshold to define the FISH spot size and Mask 2 at a low normalized intensity threshold to define the area of the sperm head as a whole using background staining. Cells with a high Mask 2 to Mask 1 area ratio tended to have well-focused FISH spots. The bivariate plot graphs the mask area ratio for the Chromosome 8 probe on the X-axis and the Chromosome Y probe on the Y-axis. Cells in the "Sperm with Tails" population from Figure 4 that have a high area ratio for both probes are back-gated on the plot in yellow. Selected cells from this population are shown enlarged in the Workspace using a composite overlay of brightfield and the two fluorescence images.

Conclusion

This study demonstrates the ability of the ImageStream 100 multispectral imaging cytometer to image cells that had been subjected to fluorescent *in situ* hybridization in suspension (FISH-IS) with chromosome specific probes.

Data are presented using the human T cell lymphoma cell line, Jurkat, and human sperm. Methodology was developed for performing FISH on these cells while maintaining them in suspension. This subsequently allowed for the hybridization to be assessed using the ImageStream 100 imaging flow cytometer.

The ImageStream 100 is capable of collecting six channels of imagery simultaneously at speeds of approximately 100 cells/second. Data files exceeding 10,000 cells can be acquired in a matter of minutes. In addition to brightfield and darkfield imagery, four image channels can be utilized with fluorescently labeled probes or fluorescent dyes. These capabilities allow the analysis of multiple FISH probes in different colors, as well as DNA binding dyes to demarcate the nuclear area, in large cell populations.

A data analysis program, IDEAS, has also been developed to optimally utilize and investigate the expanse of data. IDEAS calculates over 200 parameters from the image set associated with each cell (including all the photometric parameters used in flow cytometry) and also allows the user to define and calculate custom feature sets of particular interest. Here IDEAS is used to analyze data on Jurkat cells hybridized with a Chromosome Y probe and sperm hybridized with probes to Chromosome Y and 8. The utility of the IDEAS program, in conjunction with data containing multiple imaging modes, is demonstrated by identifying sperm cells within a heterogeneous sample using brightfield image characteristics, followed by the classification of sperm with well-focused FISH spots using a combination of fluorescence features across multiple images. Spot counting algorithms that would be of great value in FISH analysis are in development.

In conclusion, data acquisition of cells subjected to FISH-IS was performed using the ImageStream 100 platform and analyzed with IDEAS software package. The data presented here demonstrate the utility of the this technology for advancing the field of fluorescent *in situ* hybridization.