Mitotic Index Multiplexed with Apoptotic Cell Detection

**BACKGROUND AND SUMMARY**

Estimation of the mitotic index, the fraction of the cell population that is in mitosis, is an important and commonly performed assay that usually requires visual examination under a microscope. The low frequency of mitotic cells combined with manual inspection makes the assessment lengthy, tedious and often inaccurate.

The ImageStream system provides a valuable means of measuring the mitotic index of a cell population quickly and accurately. Using high resolution multispectral cell image analysis, the ImageStream can identify and quantitate cell sub-populations based on complex morphological features. In this application, mitotic cells are identified and distinguished from apoptotic cells. Because the ImageStream generates cell image data at high speed, a typical analysis can be carried out easily on tens of thousands of cells. These large numbers help ensure the statistical accuracy of the results.

In this report, 40,000 asynchronously dividing HL60 cells were stained with a fluorescent DNA binding dye and imaged to reveal nuclear morphology. A population of cells with high nuclear texture was automatically identified and found to consist of a mixture of apoptotic and mitotic cells. The apoptotic cells were independently identified by their brightfield and darkfield image characteristics and removed from the analysis, allowing the mitotic index to be measured more accurately.

**EXPERIMENTAL DESIGN AND RESULTS**

**CELL PREPARATION**

Asynchronously dividing HL60 cells were labeled with DRAQ5 for DNA content and nuclear morphology. The cells were also labeled with an anti-HLA-AF488 antibody to visualize the cell membrane, but this imagery was not used in the analysis. 40,000 cells were analyzed using the ImageStream system, producing 160,000 images in total. These images consisted of brightfield, darkfield, AF488 fluorescence, and DRAQ5 fluorescence of each cell.

**SINGLET CELL IDENTIFICATION**

Figure 1 is a plot of “Brightfield Area” vs. “Brightfield Aspect Ratio” for all the cell image objects in the data file. These features facilitate the gating of single cells from doublets, debris, and other events. The gate extended down to an aspect ratio of 0.4 (the ratio of cell width to length) so that cells in telophase would be included in the analysis. Examples of cell images from sections of the scatterplot are shown in the callouts.

After the gating, the population of “single cells” used for the analysis numbered nearly 35,000.
Identification of G2/M Phase Cells

Figure 2 presents a histogram showing nuclear staining (DRAQ5) intensity for the population of “single cells” identified in Figure 1. Cells in G2/M phase were gated within the 4N peak of the histogram, which contained approximately 6,300 cells.

Initial Identification of Mitotic Cells

Mitotic cells are identified primarily via their increased nuclear texture. The IDEAS image analysis software package, included with the ImageStream system, contains features designed to allow quantitation of textural aspects of the cell image. Two of these features, the “spot small total” and “spot medium total” were used to quantitate the fine and coarse nuclear texture, respectively. High values for one or both of these features are indicative of mitosis. Figure 3 presents a scatter plot of the features for the G2/M population gated in Figure 2. The cells gated in Figure 3 represent a “mitotic enriched” population of 734 cells.

Apoptotic Cells as an Artifact

Inspection of the “mitotic enriched” population revealed a significant fraction of apoptotic cells. A random gallery of cells from the “mitotic enriched” population, both apoptotic and non-apoptotic, are shown in Figure 4. Each cell is represented by a row of images, which, left to right, are: darkfield, brightfield, HLA-AF488, DRAQ5, and a composite overlay of the DRAQ5 and AF488 images.

The analysis of nuclear texture alone can confuse apoptotic cells with mitotic cells, whether through visual inspection or quantitative classification. Because the mitotic cell population is typically only around 1% of the sample, apoptotic cells that are mistaken for mitotic cells can lead to a high false positive rate in the mitotic index. In the next step of the analysis, apoptotic cells are identified and removed from the “mitotic enriched” population via automated analysis of the brightfield and darkfield imagery.

Identification and Removal of Apoptotic Cells

The IDEAS software package calculates a wide variety of image fea-
tures, offering multiple solutions to the classification of cells. Here, an alternative set of features that can be used to identify apoptotic cells includes high darkfield (DF) intensity, reduced cell size, and increased brightfield (BF) contrast. None of these morphological features require fluorescence staining. Examples include cells 2707, 2715, 2748, 2991, and 3264 in Figure 4.

The graph in Figure 5 plots “darkfield mean intensity” versus “brightfield area” for the “mitotic enriched” population. Cells with higher granularity -- darkfield intensity -- are typically apoptotic. A side population of cells having lower average brightfield area and higher average darkfield intensity is readily apparent. Inspection of this side population reveals that it is highly enriched for apoptotic cells.

To quantitate brightfield contrast, we used two other features: gradient RMS (or brightfield intensity gradient) and brightfield modulation. Gradient RMS is a measure of the steepness of the intensity gradients across the cell, and brightfield modulation is calculated as the difference between the darkest and brightest parts of the image, normalized by the sum of the darkest and brightest values.

Figure 6 shows a plot of these two contrast parameters for the “mitotic enriched” population. As in the case of Figure 5, a side population is evident. Visual inspection of the cell population revealed this side population to be highly enriched for apoptotic cells. A resultant apoptotic population was defined as the cells that fell into the side populations of both Figures 5 and 6.

**FINAL MITOTIC CELL IDENTIFICATION**

Figure 7 shows the apoptotic cells back-gated in red onto the “mitotic enriched” population of Figure 3.

The apoptotic classification removed 342 false positive cells from the initial 734 cells in the “mitotic enriched” population. The remaining cells were classified visually into four distinct phases of mitosis: prophase, metaphase, anaphase, and telophase. Examples of each classification are shown in Figures 8 A-D.

**CONCLUSION**

The high resolution multi-mode imagery produced by the ImageStream system provides a very detailed feature set for the characterization of mitotic index and apoptosis within an asynchronously dividing population of 40,000 cells. Further, highly automated image processing algorithms and classification tools allow the researcher to rapidly determine the precise number of cells in each stage of mitosis while simultaneously quantifying the number of cells undergoing the process of apoptosis.
Figure 8A  Prophase

Figure 8B  Metaphase

Figure 8C  Anaphase

Figure 8D  Telophase