Background: Fluorescence microscopy is an extremely useful tool to analyze the intensity, location and movement of fluorescently tagged molecules on, within or between cells. However, the technique suffers from slow image acquisition rates and limited depth of field. Confocal microscopy addresses the depth of field issue via “optical sectioning and reconstruction”, but only by further reducing the image acquisition rate to repeatedly scan the cell at multiple focal planes. In this paper we describe a technique to perform high speed, extended depth of field (EDF) imaging using a modified ImageStream® system whereby high resolution, multimode imagery from thousands of cells is collected in less than a minute with focus maintained over a 16 μm focal range.

Methods: A prototype EDF ImageStream system incorporating a Wavefront Coded™ element was used to capture imagery from fluorescently labeled beads. Bead imagery was quantitatively analyzed using photometric and morphological features to assess consistency of feature values with respect to focus position. Jurkat cells probed for chromosome Y using a fluorescence in situ hybridization in suspension protocol (FISHIS™) were used to compare standard and Wavefront Coded-based EDF imaging approaches for automated chromosome enumeration.

Results: Qualitative visual inspection of bead imagery reveals that the prototype ImageStream system with EDF maintains focus quality over a 16 μm focus range. Quantitative analysis shows the extended depth field collection mode has approximately ten-fold less variation in focus-sensitive feature values when compared with standard imaging. Automated chromosome enumeration from imagery of Jurkat cells probed using the FISHIS protocol is significantly more accurate using EDF imaging.

Conclusions: The use of EDF techniques may significantly enhance the quantitation of cell imagery, particularly in applications such as FISH, where small discrete signals must be detected over a wide focal range within the cell. © 2007 International Society for Analytical Cytology

Key terms: confocal; optical sectioning; deconvolution; time delay integration; extended depth of field; wavefront coded; point spread function; FISH
Conventional fluorescence imaging is generally much faster than confocal image stacking and can provide good spatial resolution and fluorescence sensitivity when employing high numerical aperture (NA) objectives. However, conventional fluorescence microscopy is subject to the tradeoff between NA and depth of field. As the NA is increased to improve light collection and increase spatial resolution, the depth of field is reduced by the square of the NA change. Therefore, images of weakly fluorescent signals and cellular structures located outside the ideal plane of focus can be compromised. This is most readily observed in experiments employing Fluorescence In Situ Hybridization where probes are typically under 1 μm in size, composed of a limited number of fluorescent molecules and can be distributed throughout the nucleus or cytoplasm (4). A slight defocus may preclude the detection of dim probes or cause multiple probes located in close proximity to blur into each other. Larger amounts of defocus can cause substantial blur, rendering a FISH spot unrecognizable. These tradeoffs for increased speed over the highly focused imagery produced by confocal image stacking are generally not acceptable, given that conventional microscopy, even in automated form, is still slow compared with flow cytometry. As a result, many studies of cellular phenomena employ both flow cytometry for the high throughput study of large cell populations and confocal microscopy for the detailed imaging of selected individual cells.

The ImageStream system was developed in part to address the gap between the slow but detailed information obtained by confocal microscopy and the fast but limited cellular information gleaned by flow cytometry. The ImageStream system collects six simultaneous multi-mode images (brightfield, darkfield, and up to four fluorescence colors) from cells in flow. High fluorescence sensitivity and resolution is achieved with the use of 0.75 NA optics and a 0.5 μm pixel size (5). While this platform represents a significant advance over conventional flow cytometry and standard microscopy, demanding applications such as the quantitation of FISH probed cells require imaging capabilities closer to those achieved by confocal image stacking.

The development of a prototype ImageStream system with extended depth of field (EDF) image collection described herein adds an additional benefit providing for the collection of high resolution imagery (0.75 NA and 0.5 μm pixel size) without the defocus associated with high NA optics. The EDF module combines specialized optical hardware and algorithmic post processing of the imagery to minimize the effects of defocus by bringing the entire cell into focus at the same time. Unlike confocal image stacking techniques, this new methodology acquires imagery at rates of hundreds of cells per second, allowing tens of thousands of cell images to be collected for quantitative analysis in several minutes.

In this paper we describe the methodology used in a prototype ImageStream system to significantly extend the depth of field achieving many of the characteristics of a confocal image projection. We provide a theoretical treatment of the underlying mechanism and describe the various steps involved in the generation of imagery with the entire cell in focus. We model three techniques for generating EDF imagery to assess the attributes of each and describe the selection criteria for use in the development of the prototype. We then assess the performance of the prototype using both engineered fluorescent bead samples and cells subjected to FISH.

MATERIALS AND METHODS

Bead Focus Pan

To evaluate the prototype ImageStream system with EDF imaging, a simple test was devised using 2.5 μm diameter fluorescent beads (Linear Flow Green 0.1% Intensity, Invitrogen, Carlsbad, CA). A file was collected containing imagery from nine focus positions spaced 2.0 μm apart. The ImageStream system’s autofocus control was first enabled to establish the nominal best focus for the beads. The autofocus control was then disabled and the stage was positioned at ~8.0 μm from best focus. Approximately 200 objects were imaged in darkfield and fluorescence modes at each of the nine focus positions (~8, ~6, ~4, ~2, 0, 2, 4, 6, and 8 μm from best focus), resulting in a file containing 3,600 images over a pan range of 16 μm. Two test files were collected, one using standard imaging and one using Wavefront Coded-EDF imaging.

Fluorescence In Situ Hybridization in Suspension (FISHIS)

Jurkat human lymphoma cells (ATCC, Manassas, VA) were grown in suspension culture, then fixed and permeabilized with successive incubations in 30% (30 min at 4°C) then 70% (10 min at 4°C) Carnoy’s solution (3:1 methanol:acetic acid) in PBS. After centrifugation, cells were washed once in 2X SSC, then resuspended in hybridization buffer containing the SpectrumGreen labeled chromosome Y enumeration probe according to the manufacturer’s directions (Vysis, Downers Grove, IL). To hybridize the probe, cells in PCR tubes were exposed to 80°C for 5 min and 42°C for 2 h in a DNA thermocycler. Hundred microliter of 2× SSC was added to the tubes and the cells pelleted by centrifugation. Cells were resuspended in 0.4× SSC containing 0.3% NP40 and exposed to 72°C for 2 min. The cells were centrifuged and the pellets were then resuspended in 50 μl of 1% paraformaldehyde (in PBS). The sample was then loaded into the ImageStream system and a file of 1,000 cells was collected in the standard collection mode. A second file was collected from the same sample immediately thereafter using the Wavefront Coded-EDF collection mode. Both files were analyzed in the same manner using IDEAS software (Amnis, Seattle, WA) to detect and enumerate chromosome Y in each cell. Image galleries were generated of cells having one, two or more copies of chromosome Y. Jurkat cells are well known for their cytogenetic instability.
Post Processing Deconvolution of Imagery

Imagery captured in the EDF mode was post processed using a Richardson–Lucy (R-L) iterative deconvolution algorithm to restore fidelity. Starting with a good estimate of the point spread function (PSF), the technique seeks to maximize the likelihood of the deconvolved image by using the Expectation Maximization (EM) algorithm (6). Specifically, it assumes an undistorted image \( f \) which is convolved with a PSF \( b \) where \( n \) denote the noise associated with the image. Then, EDF modified image \( g \) is given by Eq. (1).

\[
g = b \ast f + n \tag{1}
\]

where \( \ast \) is the convolution operator. The R-L algorithm attempts to reconstruct \( f \) using Eq. (2)

\[
\hat{f}_{k+1} = \hat{f}_k \left( b \ast \frac{g}{b \ast \hat{f}_k} \right) \tag{2}
\]

where \( \hat{f}_k \) is the estimate of \( f \) after \( k \) iterations and \( * \) is the correlation operator. We maintain stability and achieve convergence in 5 iterations by constraining \( \hat{f}_k \) to be non-negative and by normalizing at every step to conserve energy between \( g \) and \( \hat{f}_k \).

RESULTS

Theoretical Modeling of EDF Methodologies

Spatial resolution and depth of focus in optical systems. Diffraction causes an infinitesimally small point source of light to spread out when imaged by an optical system. The resulting intensity pattern, the Airy disk, appears as a bright central spot surrounded by a series of alternating light and dark rings (7). The intensity pattern is a projection of the PSF of the optical system onto a flat plane. A PSF that produces an Airy disk having a smaller diameter and most of its energy concentrated within the central spot results in higher spatial resolution. As objects move from the best plane of focus the Airy disk diameter increases and the energy spreads out into secondary and tertiary rings covering a larger area resulting in poorer spatial resolution. At best focus the radius \( \delta \) of the central bright spot of the Airy disk is a function the NA of the optical system and the wavelength \( \lambda \) of light comprising the image as defined in Eq. (3) (8).

\[
\delta = \frac{0.62 \lambda}{NA} \tag{3}
\]

The classical depth of field \( \Delta \), of an optical system varies inversely as the square of the NA as defined in Eq. (4) below.

\[
\Delta = \pm \frac{0.5 \lambda}{NA^2} \tag{4}
\]

For a typical moderately high resolution objective (0.75 NA) used in the center of the visible spectrum (550 nm), the diffraction limited resolution and the depth of focus as defined by Eqs. 3 and 4 are 0.45 μm and ± 0.49 μm respectively. As illustrated in Figure 1, the process of imaging is the mathematical equivalent of convolution (9). The spatial and intensity information contained within the object (250 line pair/mm bar target, upper left hand side of Fig. 1a) is convolved with the PSF of the optical system resulting in the image on the upper right hand side of Figure 1a. The image appears very similar to the object but some contrast is lost and the edges of the bars are not as sharp as in the original object. This is a result of the signal content in the original object being spread out as a result of the PSF of the optical system. The lower thumbnail images in Figure 1b demonstrate the effect of defocus on both the PSF and resulting imagery as the focus changes. At 1 μm of defocus blurring becomes evident and by 4 μm of defocus, the optical system has lost the ability to resolve the individual bars in the target. By 8 μm of defocus the bar target is unrecognizable and suffers significantly diminished intensity. Accordingly, when imaged by a 0.75 NA optical system, a cellular feature such as a FISH spot having an area of less than 1 μm and located 6 μm away from the plane of best focus will blur into an area covering more than 100 μm squared, rendering it unrecognizable to the human observer and making automated detection and enumeration difficult at best.

Confocal image stacking techniques avoid this problem by synthesizing an image of the cell with all features simultaneously in focus via the collection of multiple images of the cell at different focal planes. At each focal position an image is collected by scanning a spot of illumination over the object with a conjugate pinhole located at an intermediate image plane in the collection system. The conjugate pinhole substantially eliminates light from objects outside the focal plane, providing a crisp image of the object structures in the immediate focal plane. By applying image reconstruction algorithms to the stack of imagery, a high resolution composite image can be generated with the entire cell in focus on a single plane.

Using deconvolution to improve resolution. Within limitations, the convolution process inherent in imaging can be “undone” through post processing of the image using deconvolution. This can be visually illustrated by reversing the process shown in Figure 1a where the PSF can be “removed” via deconvolution from the image on the right to yield the object on the left. With good fore-knowledge of the PSF, deconvolution algorithms can be applied to an image to minimize the effect of optical system performance limitations, resulting in a better representation of the original spatial and intensity content of the object. This process works well where there is a high signal to noise ratio in the image and the object is a 2-D planar structure with very little depth along the optic axis, such as a semiconductor photomask, a printed page or the bar target shown in Figure 1. However, in cell analysis applications, the objects being analyzed are inherently 3-D with respect to the depth of field of the optical system. The resulting image of a cell on a detector is composed of many different degrees of point spread depending upon the location of a particular cell structure.
or probe with respect to the plane of best focus. The presence of multiple PSFs within the image substantially impairs the deconvolution process. Notwithstanding, it should be noted that 3-D deconvolution of multiple PSFs has been successfully applied to image stacks from standard fluorescence microscopes, however, the process still requires the collection of multiple images of the same cell taken at various positions along the optical axis (10).

**EDF Imaging.** EDF imaging offers an alternative method to developing a confocal-like image projection with the entire cell in focus simultaneously. EDF imaging can be accomplished at very high speed and eliminates the photo bleaching effects associated with repeated acquisitions of the cell imagery at different planes of focus. EDF imaging can be accomplished in several ways; however, the underlying principal involves the formation of a PSF that is invariant over an expected range of focal positions. For most cell imaging applications, this range is approximately 15 μm. The process of forming a PSF invariant to focal position increases the size and changes the character of the PSF when compared with the classical best focus point spread. The increased size reduces the ability of the optical system to generate contrast and resolve image detail. However, through deconvolution, the contrast can be largely restored with the benefit of providing “best-focus-like” resolution over a greatly enhanced focal range. The end result is a high resolution image of the cell with all features simultaneously in focus.

We have theoretically evaluated three methods for generation of focus invariant PSFs. The first method can be employed in concert with the time delay integration (TDI) detection methods used in the ImageStream system (11). In this method, the object plane (or detector plane) is tilted such that during the image integration process, the cell scans through a continuous range of focus positions (12). We refer to this method as Tilted Object Plane Time Delay Integration (TOPTDI). The second method involves imparting a deformation in the optical wavefront via the addition of an optical element in the aperture plane of the optical system. The deformation causes light from different focal positions from a single lateral position in object space to be imaged on the detector plane simultaneously. This method can be applied to both TDI and frame-based detection techniques and is referred to as Wavefront Coded™ (WFC) by its developer, CDM Optics (Boulder, CO) (13). A third method involves imparting spherical aberration in the wavefront by inserting a cover-glass of specific thickness between the object and the objective lens. Spherical aberration causes different regions in the aperture to focus at different points along the optical axis (14). All three methods result in a PSF which integrates light from different focal positions in object space, making it relatively insensitive to defocus. This property, in turn, enables deconvolution of the PSF to remove the spatial broadening and contrast loss inherent in the unprocessed image, thereby increasing image quality.
fidelity and creating an “in-focus” projected image of the entire cell. However, only the WFC approach allows for directed tuning of the optical wavefront to optimize the PSF for EDF imaging.

**Optical modeling of focus-invariant PSFs.** Simulations of the three methods of EDF imaging have been compared by modeling the PSF of the ImageStream optical system and incorporating the perturbations associated with each EDF method. Through-focus contrast plots were generated over a range of −10 to +10 μm of defocus to compare the performance of each of the methods to standard imaging. Synthetic imagery of a point source was generated for each of the four cases (standard, TOPTDI, WFC, and induced spherical aberration) at best focus and for a position 5 μm from the plane of best focus. Because noise can be amplified during the deconvolution process, adversely affecting image quality; random noise, consistent with the noise present in the ImageStream system (σ = 1 count), was added to the imagery to simulate real-world conditions. For each EDF method, a kernel composed of the PSF at best focus was deconvolved from the imagery to simulate the entire process of image collection, wavefront perturbation and deconvolution in the ImageStream system. The peak intensity for each case was then computed for both pre-processed and post-processed imagery and compared. These simulations provided insight into the benefits and limitations of each method.

To simulate the spherical aberration-EDF approach, the ImageStream optical system was modeled with a decreased flow cuvette thickness to add 1.8 waves of peak to peak spherical aberration after refocusing. This optical configuration was then used to model the PSF at the various focus locations required for subsequent analysis.

To simulate the WFC-EDF approach, a phase plate consisting of an optically clear element having an orthogonally crossed two-axis cubic waveform was modeled. The slight variations in thickness across the plate’s surface, the element’s Sag, serve to retard or advance the phase of the wavefront. From a geometric perspective, the angular changes in the surface of the element cause ray paths to deviate from their original course to image light from different points along the optic axis for a given lateral position in the image plane. For this simulation, we modeled an element with a Sag of the form shown in Eq. (5), where \( n = 3 \).

\[
Sag = \sum_{n=[3.79]} a_n \left( \frac{x}{r_0} \right)^2 + \left( \frac{y}{r_0} \right)^n
\]  

(5)

A coefficient \( a_n = 0.000122 \) was selected to generate approximately five waves of peak to valley wavefront error over the aperture. A 3-D rendering of the element is shown in Figure 2 containing about 6.6 μm of total sag. The element was modeled at an exposed aperture stop in the ImageStream optical system to generate the PSFs used in the subsequent analysis.

Simulation of the TOPTDI-EDF method was slightly more complicated because, unlike the other methods, the resulting PSF for a single focal position in the image cannot be computed from a single focal position in object space. In TOPTDI the object being imaged is tracked by the TDI detector over an extended field of view and the object’s trajectory is not co-planar with the detector. Therefore the PSF for any point in the final image is an integration of all PSFs accumulated from many focal positions as the object traverses the field of view. Multiple PSFs must be modeled at discrete lateral and axial locations corresponding to the path an object traverses along a tilted object plane during the image integration process and summed into a composite, normalized PSF. The object plane of the ImageStream optical model was tilted such that an object flowing through the field of view traversed a distance of 10 μm along the optical axis during its lateral traversal of a 256 μm field of view. Discrete PSFs were modeled at seven locations across the field:

1. 128 μm field height (top of field), +5.00 μm from plane of best focus
2. 85 μm field height, +3.33 μm from plane of best focus
3. 42.7 μm field height, +1.67 μm from plane of best focus
4. center of field, within the plane of best focus
5. −42.7 μm field height, −1.67 μm from plane of best focus
6. −85 μm field height, −3.33 μm from plane of best focus
7. −128 μm field height (bottom of field), −5.00 μm from plane of best focus

In the same manner, additional composite PSFs were generated for other focus positions by modeling discrete PSFs for objects starting at +10 μm of defocus (at the 128 μm field height) and traversing through to 0 μm of defocus (at the −128 μm field height position). Likewise PSFs were modeled for objects starting at a 0 focus.
position at the top of the field and traversing through to −10 μm at the bottom of the field. These composite PSFs were used to synthesize effective PSFs for focused and defocused locations used in the subsequent analysis.

**Evaluation of modeled focus-invariant PSFs.** A convenient method to theoretically evaluate the expected performance of the various EDF approaches is to compare their modulation transfer functions (MTF). The typical MTF plot provides a quantitative assessment of contrast over a range of spatial frequencies (15). For the comparison of EDF approaches, a single spatial frequency was chosen and curves were generated for different focus positions. A through-focus MTF plot shows the behavior of the decreasing contrast function on either side of the best focus position. The ImageStream system utilizes a detector pixel size of 18 μm, corresponding to a maximum sampled spatial frequency of 27.8 lp/mm at the detector plane. The through-focus MTF plots were calculated at approximately half the maximum resolvable spatial frequency, or 14 linepairs/mm (500 lp/mm in object space), over a focal range of ±10 μm in object space. The optimal performance for an ideal system would be a flat response (i.e. constant MTF) with maximum contrast over the widest focal depth. Figure 3 shows a family of curves consisting of the contrast versus focus for the various EDF methods (without PSF deconvolution), as well as the non-EDF optical system.

As shown in Figure 3, the standard non-EDF system (solid line) provides the best contrast at the plane of focus. However, the contrast falls off rapidly as the focal position changes. At 2.5 μm of defocus, the standard system provides no contrast in the image. Numerous null contrast points are observed throughout the plotted focal range. The TOPTDI (continuous long dash) system integrates light from focal planes over a range of −5 to +5 μm from best focus. At best focus the contrast is about 0.2, which is less than one-third of the standard in-focus contrast, but the TOPTDI contrast remains relatively constant over a much wider range of focal positions. The WFC method provides slightly lower contrast than the TOPTDI approach, but with a greater enhancement to the depth of focus. The spherical aberration method sacrifices more contrast than either of the other EDF methods modeled here, while providing less improvement to the depth of field. It also exhibits a classical nonsymmetrical behavior about the best focus position. The lower plot in Figure 3 provides a “modulation normalized” view of the same data which more clearly shows the relative depth of field enhancements provided by each method.

Figure 4 shows simulated point source imagery generated for the various EDF methods using collection parameters associated with the ImageStream system, including an 18 μm pixel size (0.5 μm in object space), 0.75 counts of random noise per pixel, and the corresponding PSFs for the various focus positions. Figure 4 also shows the results of ten iterations of a Richardson–Lucy deconvolution algorithm applied to each of the EDF images using the best focus PSF as the deconvolution kernel (16). The peak intensity (based on 10 bit A/D conversion) for each thumbnail image is listed above each image. As demonstrated in the upper thumbnail images in Figure 4, the PSF deconvolution process can recover a high degree of image contrast (673 counts for TOPTDI 5 μm of defocus versus 801 counts at best focus in the non-EDF image). The display of each image is scaled for visualization purposes, so that the brightest pixel in the image appears white, and the lowest intensity in the background appears black, causing the background noise to appear higher in the cases where the PSF imagery has a lower peak intensity, particularly in the case of standard imagery at 5 μm of defocus. It should be understood in all cases the noise level in all pre-deconvolved imagery is the same.

The simulated imagery illustrates the effectiveness of both the TOPTDI and WFC methods in maintaining a constant PSF over an extended focal range. The results are particularly striking when comparing the deconvolved EDF imagery to the standard imagery at 5 μm of defocus. The peak intensity in the standard image drops to 19 counts (including 10 count background level) at the defocus position while both the TOPTDI and WFC methods produce peak intensities in excess of 350 counts, resulting in an increase in contrast of more than 30-fold (340 counts vs. 9 counts) for both of these EDF methods over standard imaging. The imagery in the WFC method exhibits some...
asymmetrical horizontal and vertical artifacts after processing. However, the artifacts are attenuated by more than an order of magnitude in comparison to the primary image. Optimization of this first-generation WFC element and deconvolution kernel is expected to further reduce these artifacts. The induced spherical aberration approach fares better under defocus conditions than the standard optical system, but exhibits much lower contrast with defocus than the other two EDF methods (151 counts vs. ~350 counts).

**PSF measurement and generation of the deconvolution kernel.** Simulation of the various EDF methodologies described above offered insights into the advantages and disadvantages of each of the alternative approaches. The WFC method was chosen for implementation in the EDF ImageStream system based on its high level of

---

*Cytometry Part A* DOI 10.1002/cyto.a

---

**BEST FOCUS PSF (Pre and Post Deconvolution)**

<table>
<thead>
<tr>
<th>Standard Imaging</th>
<th>TOPTDI</th>
<th>WFC</th>
<th>Sph. Aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>801</td>
<td>203</td>
<td>136</td>
<td>106</td>
</tr>
</tbody>
</table>

![Image of BEST FOCUS PSF](image1.png)

**After Deconvolution**

<table>
<thead>
<tr>
<th>Standard Imaging</th>
<th>TOPTDI</th>
<th>WFC</th>
<th>Sph. Aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>673</td>
<td>504</td>
<td>565</td>
<td></td>
</tr>
</tbody>
</table>

**FIVE MICRONS OF DEFOCUS (Pre and Post Deconvolution)**

<table>
<thead>
<tr>
<th>Standard Imaging</th>
<th>TOPTDI</th>
<th>WFC</th>
<th>Sph. Aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>109</td>
<td>144</td>
<td>31</td>
</tr>
</tbody>
</table>

![Image of FIVE MICRONS OF DEFOCUS](image2.png)

**After Deconvolution**

<table>
<thead>
<tr>
<th>Standard Imaging</th>
<th>TOPTDI</th>
<th>WFC</th>
<th>Sph. Aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>351</td>
<td>353</td>
<td>151</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 4.** Synthetic “point” imagery for standard and EDF imaging at best focus and 5 μm away from best focus. Imagery is shown pre and post deconvolution with peak intensities shown in upper left corner of each image. TOPTDI and WFC methods provide 18 times higher intensity than standard imaging at 5 μm of defocus.
performance in modeling, its flexibility in implementation and that it offers the ability to custom tailor the wavefront specific applications.

To implement and empirically test the WFC method, a crossed cubic WFC element was procured (CDM Optics, Boulder, CO). The element was installed at an external aperture stop in the ImageStream optical system and the PSF measured by running a sample of 200 nanometer diameter fluorescent beads (Invitrogen, Fluospheres® carboxylate-modified microspheres, 0.2 μm, yellow-green, 505/515 2% solids, F-8811) was prepared at a 10,000:1 dilution and run on the ImageStream EDF system. These beads are sufficiently small relative to the pixel size and diffraction limited spot size of the ImageStream optics to be treated as point sources. Figure 5 shows a small sampling of the PSF imagery collected during the run. The form of the PSF corresponds very closely to the modeled imagery shown in Figure 4. Approximately 1,000 bead images were collected and processed to generate the composite PSF shown in the lower right of Figure 5. Processing included a linear interpolation of each image to remove shift variance caused by under-sampling and then spatially aligning each image such that the highest intensity appeared in the same pixel for each image. A mean intensity was then calculated for each pixel to generate the composite PSF used in the subsequent image deconvolution step.

**Empirical Testing of ImageStream with EDF**

**WFC imagery from bead focus pan.** Figure 6 shows an image gallery of 18 beads comprised of two beads selected consecutively from each interval of the focus pan. Darkfield (blue) and fluorescence images (green) are shown for each bead with a gallery of standard imagery on the left and EDF imagery on the right. The EDF imagery clearly maintains a much higher degree of focus throughout the pan range. Focus blur is present in the standard imagery at ±2 μm of defocus in both darkfield and fluorescent images (objects 701, 702, 1102, 1103). At ±4 μm of defocus blurring is significant (objects 500, 501, 1,306, 1,307) and the bead imagery exhibits a marked decrease in peak intensity as well as a large change in apparent area. By ±8 μm of defocus, bead images (objects 100, 101, 1,707, 1,708) become difficult to discriminate from the background.

In marked contrast, the WFC-based EDF imagery maintains consistent image characteristics throughout the focus pan with both the bead area and intensity remaining relatively constant throughout the pan. This is particularly evident in the green fluorescent bead imagery in the right hand column. There are some artifacts present at higher levels of defocus in the form of horizontal and vertical lines emanating from the primary bead image and directed toward the top and right hand side of the page. The artifacts largely resemble those generated in the simulation and exhibit much lower intensity than the primary image. These artifacts are a result of slight changes to the PSF with focus and can be minimized with optimizations of the WFC element and deconvolution kernel. Modeling of the nonorthogonal nature of the artifacts has shown that they are also due in part to residual uncorrected spherical aberration in the optical system. The darkfield imagery appears similar in nature to the fluorescence imagery, however, it exhibits stronger deconvolution artifacts especially at high levels of defocus. This may be due in part to the fact that the deconvolution kernel was generated from the fluorescent imagery. Future optimizations will include channel specific kernels, balancing of in focus and out of focus imagery for kernel generation, elimination of residual spherical aberration and optimized WFC waveforms; all of which will reduce artifacts.

Figure 7 provides a quantitative analysis of the entire image set from which the imagery in Figure 6 was selected. In this Figure the peak pixel intensity and area of each object is plotted vs. object number for both the standard and EDF image sets where each dot in a plot represents a single bead. A total of 1,800 objects were imaged, consisting of approximately 200 objects acquired at each of nine focal positions with each focal position separated by 2 μm. Object 1 and object 1,800 are therefore spaced 16 μm apart with the best focus position corresponding to object numbers in the range of 800–1,000. The best focus position was gated using the regions “In Focus pk” or “Infocus Area” with the accompanying mean values for the gated data shown below the plot. In a similar manner, beads from the ±8 μm focus positions were also gated.
Referring to the upper plots in Figure 7, the standard image set (left) exhibited close to a 14-fold decrease in average peak intensity (641 counts vs. 44 counts) between the best focus and the “8 μm defocus” positions. In contrast, the EDF plot (right) showed only a 2-fold decrease in average peak intensity (613 counts vs. 287 counts) over the same focus pan. Allowing for the increased focal range and the larger bead size, these results were consistent with the theoretical models for the behavior of peak intensity.

Referring to the lower plots in Figure 7, the fluorescent EDF imagery exhibited a consistent area of approximately 24 pixels throughout most of the focus range, rising to 32 pixels at the +8 μm defocus position. In contrast, the standard imagery exhibited an increase in area of almost 14X from 32 pixels to 437 pixels at −8 μm of defocus. Using the area and peak intensity as figures of merit, the ImageStream with EDF imaging demonstrates seven to fourteen times better feature consistency through a depth of field covering the majority of most prokaryotic and eukaryotic cell diameters.

A statistical analysis of the noise contained in the imagery was performed by evaluating the standard deviation in background signal outside the bead image for each individual object. An analysis of over 2100 objects for each focus pan indicates the median standard deviation in

**Fig. 6.** ImageStream standard (left panel) and EDF (right panel) imagery collected over a 16 μm focus pan. Left and right panels show both dark-field (blue) and fluorescence (green) imagery. Standard image collection exhibits significant blur while EDF imagery maintains size and intensity throughout the entire 16 μm focus pan.
background signal is 0.97 and 1.30 counts respectively for the standard and EDF focus pans (σ listed in upper plots of Fig. 7). The increase in noise of 0.33 counts will degrade the Signal to Noise ratio and therefore negatively impact the sensitivity of the instrument. However, in comparison to standard imaging, the degradation will be greatest for objects located at the best plane of focus. For objects located away from the best plane of focus, the increased signal maintained via EDF should more than offset the increase in noise. Sensitivity studies of the standard ImageStream demonstrate sensitivity superior to standard flow cytometry for fluorochrome conjugated 3 μm particles and indicate an ability to detect as little as 50 molecules of equivalent soluble fluorescein (17). Future work with EDF imaging will include a detailed sensitivity study of the EDF collection mode.

Comparison of EDF and standard imaging for chromosome enumeration. Automated chromosome enumeration via FISH is an application for which EDF imaging may confer significant benefits. Defocus causes

![Fig. 7. Comparison of Peak Intensity and Area feature variation for standard and EDF imaging modes of 2.5 μm beads collected over a 16 μm focus range. Dot plots show feature values (FL1 Peak Intensity or Area) plotted against focus position (object number). EDF image collection demonstrates 7-14 times less variation over the tested focal range.](image-url)
significant changes in the presentation of probes often blurring one into another or spreading out the signal to such a degree that it is difficult to automatically segment, or visually separate FISH probes from each other or from nonspecific background binding in the nucleus.

To compare the efficacy of chromosome enumeration between the standard and EDF ImageStream configurations, a sample of Jurkat cells was hybridized to a Chromosome Y probe as described in the Materials and Methods section. The results were automatically analyzed to enumerate copies of the Y chromosome in each cell. Simple classifiers using brightfield imagery were developed to exclude cellular debris, doublet events and other artifacts from the analysis. A specialized segmentation routine and connected components analysis was performed on the fluorescence imagery to generate a first pass enumeration of chromosomes on single cells (18,19). A refinement of the monosomy and disomy classified cells from the first pass enumeration was performed to eliminate false positive events. After the final classification step, the resulting imagery was manually reviewed to qualitatively judge the efficacy of the final classification. This analysis was not intended to be a rigorous examination of the efficacy of the ImageStream with EDF for chromosome enumeration. Rather, this experiment is an extension of the previous work discussed in this paper and was performed to explore an application for which the ImageStream with WFC-EDF imaging may have a beneficial result.

After preparation the sample was loaded into an ImageStream system with a prototype WFC module enabling the EDF collection mode. A file containing 1,000 cells was collected in the standard configuration. The ImageStream was reconfigured for EDF collection and an additional 1,000 cell file was collected. The entire collection time for both files was several minutes (including the time switch from standard to EDF modes). The files were then analyzed using the IDEAS cell analysis program to enumerate chromosomes in each cell in each sample. Visual observation of the resulting monosomy and disomy populations was used to confirm automatic classifier results and to gain insights into the cause of performance differences between the two collection modes.

Figures 8–12 present a brief overview of the analysis of these files. Figure 8 shows a sampling of 20 cells (10 in each collection mode) highlighting some of the challenges in the classification of disomies within the Jurkat sample. The FISH probe imagery is superimposed over a reduced contrast brightfield image of the cells to provide a sense of scale and verify that the probes are located within the cell. In the upper panel, cell imagery, collected in the standard imaging mode, shows that at least one of the FISH probes is positioned out of the plane of focus. Conse-
quently, the area of the out of focus probe increases and sometimes engulfs the second probe in the cell. Like the bead imagery shown in Figure 6, the intensity falls off significantly with defocus, making it difficult to automatically segment the probe or even see it by eye. In at least one case, object 916, two probes appear to be in close proximity. Slight defocus may have caused these relatively bright probe images to blur into each other, creating what appears to be a single large probe. Although it cannot be specified with certainty, this cell is thought to have two probes due to the total intensity of the probe signal and the elongated shape of the probe.
Fig. 10. (Upper) Histograms showing first pass enumeration results for monosomy, disomy and polysomy cells for both standard and EDF collection modes. (Middle) Refinement of monosomy populations using peak intensity and area to eliminate false positive events. (Lower) Refinement of disomy populations to eliminate false positive events. EDF imaging isolates 2-fold more true disomy positive cells and provides clearer differentiation between true positive and false positive events.
EDF imagery presented in the lower half of the figure shows discrete FISH spots even when they are positioned in close proximity to each other. Unlike the standard collection mode, the EDF imagery exhibits no blurring of FISH spots. This is also readily apparent when comparing the larger selection of images shown in Figures 11 and 12. Each FISH labeled chromosome appears as a bright, tightly focused spot.

Development of FISH spot enumeration classifier and classification results. To determine the efficacy of EDF imaging on the enumeration of chromosomes, a simple five step classifier was developed using the IDEAS analysis software. The first two steps involved segmentation and the selection of appropriate objects within the data file for subsequent analysis (16). Object selection was accomplished by plotting the brightfield aspect ratio vs. brightfield area as shown in the dot plot in Figure 9. A gate was drawn that encompassed primarily single cells (example object No. 424) and excluded cell fragments/debris (example object No. 845) and groupings of cells (example object No. 75). The gate defined a population named “Cells” containing 595 individual objects, to which...
subsequent analysis was applied. A similar segmentation and selection process was performed on the standard collection file and resulted in 588 individual objects.

The third step in classification, shown in the lower portion of Figure 9, involved refinement of the standard segmentation mask to isolate areas of local maxima in each fluorescence cell image. A fluorescence image of object 89, collected in Channel 3, is shown prior to segmentation (a), after initial segmentation (light blue overlay) to identify all areas containing light above background (b), and after Morphology segmentation (light blue overlay), a form of contour masking to identify areas of local maxima contained in the initial segmentation mask (c).

The fourth step in classification employed an IDEAS feature called “FISH Spots” which used the Morphology mask to perform a connected components analysis to enumerate discrete spots contained within each fluorescent image. The results of this computation and the final gating of disomic cells, the fifth step in the classification, are shown in Figure 10. As shown in the FISH Spot enumeration histograms at the top of Figure 10, the first pass analysis using the standard collection mode yielded enumerations of 506 monosomic, 67 disomic and 15...
polysonic (three or more spots) cells. In contrast, the first pass enumeration with EDF imaging yielded 421 monosomic, 136 disomic and 58 polysonic cells. The EDF collection mode therefore produced a 2× increase in the number of disomy and polysony-classified cells, with a 17% decrease in monosomic cells. Manual review of the monosomy and disomy-classified cells in both collection modes revealed a significant number of false classifications where hybridization had failed, leaving only nonspecific binding in the nucleus.

To improve classification accuracy, each population of monosomy and disomy-classified cells was further analyzed by plotting peak intensity vs. area for the fluorescence channel. Nonspecific binding generally has low peak intensity and large area and is therefore plots of peak intensity vs. area provide for good discrimination of nonspecific binding events. Bivariate plots of this analysis are shown in the middle and lower plots of Figure 10. The discrimination boundaries for the standard collection mode are not clear. This is most evident in the boundary drawn to discriminate true and false positives for disomy refinement (lower left plot of Fig. 10). The boundary is complex and arbitrary and unsuitable for fully automated classification. In contrast, the boundaries drawn for the EDF analysis are clear with the true and false positive populations showing excellent separation. Minor shifts in feature values due to preparation differences or instrument variations will not significantly affect the results of the classifications making these features and boundaries suitable for fully automated classification. The refined classifications result in 45 and 96 disomy events for the standard and EDF collection modes respectively and a respective 191 and 189 monosomy events.

Figures 11 and 12 display a random set of images from each set of “Refined” and “False Positive” populations defined in Figure 10. A review of this imagery sheds light on why the EDF collection mode exhibits a 2-fold increase in discrimination of disomic events. First, the EDF collection mode largely eliminated focus variation, producing small, bright, tightly focused spots for each hybridization event as evident in both “Refined” monosomy and disomy populations (upper left hand galleries in Figs. 11 and 12). Second, these tightly focused spots dramatically improved the performance of the morphological segmentation algorithm and final classification steps by forming a clear demarcation between well-hybridized probes and nonspecific binding events (center and lower right hand side of Fig. 10). The false positive events shown in the lower right hand side galleries of Figures 11 and 12 are a result of nonspecific binding and result in large segmentation masks with low peak intensities. The use of peak intensity and FISH Spot area effectively discriminates false positive events. By contrast, it is very difficult to discriminate between nonspecific binding and highly defocused probes as shown in the lower left hand galleries of Figures 11 and 12. Third, by eliminating focus variation, probes located away from the ideal focal plane still appear as small spots. This is in contrast to the probe imagery found in cell numbers 213, 228, 245, 255, 257, 275 etc. shown in the upper left hand gallery of Figure 11 or cell numbers 15, 251, 279, 465 and 624 etc. in the upper left hand gallery of Figure 12. Tight focus substantially reduces the probability of events where a defocused probe image engulfs or contacts a second probe image in the cell. With standard imaging it is a rarity to find imagery similar to cells Nos. 55 and 247 from Figure 12, where two probes are in close proximity and tightly focused. More likely than not, one of these probes will blur, engulfing the other leading to a misclassification of a disomic event as a monosomic event.

**DISCUSSION**

The collection of multimode imagery provides a comprehensive feature set to more effectively eliminate artifacts and allow for the complex analysis of the location, distribution and translocation of biomarkers. However, standard, nonconfocal approaches to image collection are hindered by depth of field limitations. The EDF capability described herein modifies the ImageStream optical system with an element in aperture space to alter the wavefront in a deterministic way. The combination of a modified wavefront and post processing of the imagery helps to mitigate the spatial resolution loss and blurring associated with defocus. The result is a 2-D projection of the 3-D cell for each of six multimode images acquired at rates 100–1,000 times faster than confocal image stacking techniques. With the WFC-EDF enhancement, micron-level spatial resolution can be maintained over the entire cell so cellular structures and probes lying outside the plane of best focus can be analyzed with greater accuracy, as demonstrated with the FISH application. This technology may also be applied to other applications such as transcription factor translocation and protein co-localization (20, 21).

While the elimination of focus variation in a 2-D projection of a cell will likely be beneficial in many applications, it may be limiting in others, such as co-localization assays. The key possibility was a key consideration in the choice of the WFC methodology for the implementation on the EDF-modified ImageStream, since the WFC approach can be implemented to different degrees or disabled completely with the exchange of different elements in the system’s aperture plane. In future studies, applications will be analyzed in both standard and WFC-EDF imaging modes to evaluate the utility of employing WFC-EDF.

Although the results presented in the paper are at a prototype stage, they present a compelling picture for the potential of the ImageStream system with WFC EDF imaging. Future optimizations will provide for further improvements in image quality and advanced capabilities. Since the ImageStream collects imagery in a flow cuvette with image collection access to all four sides of the cell, unlike slide-based imaging systems, there exists the potential to develop a two axis orthogonal implementation of the architecture described herein. Coupling the ImageStream architecture with WFC in this manner would provide a means to perform full 3-D cell mapping and optical sectioning similar to confocal techniques, but at
two to three orders of magnitude greater speed and without photobleaching. Each axis would collect an EDF projection of the cell from orthogonal perspectives, allowing a 3-D reconstruction of the cell as is done in optical topographic methods. However, unlike confocal techniques, this method would provide an isometric perspective with consistent resolution in all axes. Since the cells would be mapped in a single pass, photobleaching would be minimized and, with sufficient image processing capacity, tens of thousands of cells could be analyzed in several minutes.

**LITERATURE CITED**