Sensitivity Measurement and Compensation in Spectral Imaging

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Received 16 November 2005; Revision Received 5 April 2006; Accepted 17 April 2006

Background: The ImageStream system combines advances in CCD technologies with a novel optical architecture for high sensitivity and multispectral imaging of cells in flow. The sensitivity and dynamic range as well as a methodology for spectral compensation of imagery is presented.

Methods: Multicolored fluorescent beads were run on the ImageStream and a flow cytometer. Four single color fluorescent control samples of cells were run to quantify spectral overlap. An additional sample, labeled with all colors was run and compensated in six spectral channels.

Results: Analysis of empirical data for sensitivity and dynamic range matched theoretical predictions. The ImageStream system demonstrated fluorescence sensitivity comparable to a PMT-based flow cytometer. A methodology for addressing spectral overlap, individual pixel anomalies, and multiple imaging modalities was demonstrated for spectral compensation of K562 cells. Imagery is shown pre- and post-compensation.

Conclusions: Unlike intensity measurements made with conventional flow cytometers, object size impacts both dynamic range and fluorescence sensitivity in systems that utilize pixilated detection. Simultaneous imaging of alternate modalities can be employed to increase fluorescent sensitivity. Effective compensation of complex multimode imagery spanning six spectral bands is accomplished in a semi-automated manner.

Key terms: multispectral imaging; fluorescence intensity; spectral compensation

The ImageStream multispectral imaging flow cytometer is a charge-coupled detector (CCD)-based instrument that acquires six simultaneous images of individual cells directly in flow. The six images are formed in six different spectral bands across the visible spectrum, each spanning ~50 nm. Each of the six images is in spatial register with its counterparts, meaning that the light emitted, scattered, or transmitted at every location in the object is independently measured as a pixel intensity in each of the six spectral bands. Simultaneous image modes include brightfield (transmitted light), darkfield (side scatter), and four channels of fluorescence.

Operation of the ImageStream system is similar to flow cytometry and the imagery can be used not only to measure numerous morphologic characteristics of each cell, but also any of the fluorescence intensity-based measurements produced by standard flow cytometers. Proper compensation of spectral crosstalk is essential for the quantitation of image-based data. The compensation process, while conceptually similar to compensation of flow cytometric data (1), involves several additional data processing steps, which are described.

Once the data are compensated, fluorescence intensity measurements can be made from the imagery. Unlike the situation in conventional flow cytometers, in an imaging system the object size has a bearing on the detection limit, dynamic range, and intensity resolution of the system. The size-dependence of these instrument characteristics is described and a simple method is proposed for the comparison of intensity resolution between instruments. The proposed method is independent of object size or the instrument architecture, whether imaging or nonimaging.

MATERIALS AND METHODS

Instrumentation

The layout and key operational components of the ImageStream flow imaging cytometer have been described previously (2–4). Briefly, hydrodynamically focused cells are illuminated with a 488 nm laser oriented perpendicular to the collection axis while simultaneously being trans-illuminated along the collection axis by a brightfield light source. Light is collected with an imaging objective lens and projected on a CCD operating in time-delay integra-

Grant sponsor: NIH; Grant numbers: 9 R44 CA01798-02, 1 R43 GM58956-01
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Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20306
tion (TDI) mode (5–7), which is a method of electronically tracking the cells to increase the integration time of the detector while preserving image resolution. Unlike conventional flow cytometry (8), TDI operation requires no triggering, involves no dead time, and can image multiple objects in the field of view simultaneously. As a result, all objects in the core are imaged, there are no coincidence issues during detection, and the typical integration time of several milliseconds per cell is independent of the imaging rate, which can exceed 15,000 cells per minute. Prior to projection on the CCD, the light is passed through a multispectral optical system that directs six different colors of light to six different locations across the detector, perpendicular to the axis of flow. With this technique, light collected from each cell is decomposed into a set of six images, each corresponding to a different spectral band, and spatially offset from the other images of the same cell to facilitate image processing and quantitation.

**Antibodies and Immunofluorescent Staining of Cells for Compensation Studies**

Anti-CD45 mAb conjugated with PE AF610 was obtained from Caltag (Burlingame, CA), purified anti-CD71 was obtained from Caltag and conjugated with Cy3 using a commercial conjugation kit (Amersham BioSciences, Piscataway, NJ). FITC-conjugated anti-NFkB (p65) was obtained from Santa Cruz Biotech (Santa Cruz, CA).

For four color staining, K562 human erythroleukemia cells (ATCC, Manassas, VA) were incubated for 30 min at 4°C with both PE AF610-conjugated anti-CD45 mAb and Cy3-conjugated anti-CD71. After a wash in PBS with 0.1% FBS, the cells were resuspended in 0.5 ml of 4% PFA and incubated for 10 min at room temperature. After washing, the cells were resuspended in 0.1 ml of PBS containing 0.2% Triton X 100 and 0.2% FCS. An appropriate dilution of FITC-conjugated anti-NFkB (p65) was added to the cells and incubated for 30 min at 4°C. After washing, cells were resuspended in 0.1 ml of PBS/2% PFA and DRAQ5 nuclear dye (BioStatus, Leicestershire, UK) added at a final concentration of 50 μM.

For single color control populations, K562 cells were processed as above, but stained with a single antibody only. A separate cell sample was stained with DRAQ5 only. PFA fixed, antibody stained cells were pooled and imaged acquired without bright field illumination on the ImageStream using a laser power of 200 mW. A separate aliquot of cells was stained with DRAQ5 only and run on the ImageStream. These DRAQ5-stained cells cannot be pooled with the antibody-stained cells because the DRAQ5 dye will stain those as well. Control image files were merged, compensated, and analyzed post-acquisition using the ImageStream Data Analysis and Exploration Software™ (IDEAS™) developed by Amnis.

**Sensitivity Comparison Measurement with Fluorescent Beads**

Sphero 6 peak Rainbow calibration particles of 3 and 6.3 μm diameter were obtained from BD Biosciences (San Jose, CA). Beads from a single sample were split and analyzed on the ImageStream and a FACS Canto™ (BD Biosciences). ImageStream data were analyzed using the IDEAS image analysis software. FACS Canto™ data was collected under normal instrument configuration with voltage settings optimized for separation between the blank and 1st peak in the bead set. Uncompensated FACS data was subsequently analyzed using FlowJo analysis software (Tree Star, Ashland, OR).

**RESULTS**

**Spectral Crosstalk Compensation Process**

Accurate spectral compensation of ImageStream data is critical for the quantitation of cell morphology, optical density, texture, and the amount and distribution of fluorescent probes on or in the cells of interest. Poor compensation can compromise the spatial integrity of the data and degrade sensitivity, thereby reducing the efficacy of these measurements. Like compensation in conventional flow cytometry, spectral compensation of multispectral imagery requires the application of a crosstalk compensation matrix (9). Unlike conventional flow cytometry, the compensation process in a multispectral imaging system involves the added dimension of space, so the compensation matrix is applied on a pixel-by-pixel basis. Prior to the application of the matrix, it is essential that there be accurate spatial registration between the six different channels. Without accurate registration, fluorescence detected at a particular pixel location in an image in one channel will be compensated at the incorrect location in the other channels. Further, since each pixel on the detector has its own characteristic noise, dark current offset, and gain (10,11), the spectral compensation process must address the variations in CCD uniformity. Finally, since the image set associated with each cell generally includes brightfield and darkfield images (12), the compensation procedure must handle these imaging modes appropriately. Despite these additional considerations, spectral compensation of multispectral/multimodal imagery is highly effective and can be automated to the same degree as in conventional flow cytometry.

The multispectral image compensation process employed in the ImageStream system includes 10 steps as listed below:

1. Dark current pixel offset measurement
2. Pixel gain measurement
3. Inter-image spatial offset measurement
4. Application of dark current corrections
5. Application of spatial alignment corrections
6. Fluorescence crosstalk measurement
7. Brightfield cross talk measurement
8. Calculation of compensation matrix
9. Application of compensation matrix
10. Application of brightfield pixel gain compensation

All steps in the process are highly automated but allow for user intervention, if desired.
Dark Current, Gain, and Spatial Offset Measurement and Correction

Each pixel on a CCD detector has a characteristic baseline output, known as its dark current offset, and a response to light exposure, known as the pixel gain. It is generally useful in quantitative imaging applications to correct pixel gain and dark current offset variations from pixel to pixel. The ImageStream’s CCD detector is operated in TDI mode, so the signal from each cell is transferred from row to row down the entire height of the 512 row detector, in synchrony with the motion of the cell, before reading the imagery off the bottom of the chip. As a result, dark current offsets are spatially averaged over all 512 rows of the CCD and are typically less than three counts per pixel ($\sigma \sim 0.75$ counts). Despite the relatively small absolute adjustment, performing the dark current offset correction greatly enhances the ability of the data analysis software to detect faint fluorescence signals by providing an extremely uniform background level. During the daily automatic calibration routine, the average dark current offset values (in counts) from each pixel column of the TDI detector are measured in the absence of any illumination and the deviation from the nominal value of 30 counts per pixel is stored with each data file generated by the system.

Just as with dark current offset, TDI operation minimizes variations in pixel gain, which is the rate at which the pixel value changes with changes in light exposure. Nevertheless, the ImageStream compensation process incorporates a gain correction step primarily as a means to correct for nonuniformities in brightfield illumination. The system automatically calibrates pixel gain by turning on brightfield illumination and adjusting the average intensity value to a target value (typically 200 counts), as measured over all pixels in a row of image data. The gain correction factor for each pixel is then calculated as the multiplicative factor necessary to adjust that pixel to the average value. When a data file is generated, the ImageStream automatically appends the appropriate gain factors to the file to be used for brightfield gain correction. All pixel gain and dark current offset values are automatically checked against known limits to detect faulty pixels and/or optical alignment errors. Together, the pixel gain and dark current offset corrections ensure a consistent and accurate photometric response for each pixel in the image (13).

Spatial registration errors between the various image channels are measured by imaging the same object in all six channels simultaneously and comparing the location of the images in each channel. During the automated daily calibration routine, the system is temporarily configured to produce six simultaneous brightfield images of the system’s calibration beads. The vertical and horizontal spatial offsets between the images of each bead are computed relative to a reference image (channel 6) by performing a two axis auto-correlation (14), which generates a high resolution sub-pixel alignment value for each axis and each channel. The calculation is repeated for each of ~500 beads and the averaged offset values are stored with each data file for use during the automated compensation process.

Fluorescence and Brightfield Crosstalk Measurement

Though the image-based compensation calibrations described above are different from those employed in the compensation of standard flow cytometric data, they are performed automatically by the system and therefore don’t significantly change the compensation workflow familiar to flow cytometrists. The remainder of the ImageStream compensation procedure is very similar to that used in standard flow cytometry in that the ImageStream data analysis software can utilize compensation matrices from prior experiments or can calculate a matrix from singly-labeled cells imaged without brightfield. The singly-labeled cells can be pooled in a single sample, as long as each cell is either unlabeled or singly labeled with only one probe (Fig. 1). Separate control samples can also be run individually in cases where probes may stain other cells in the mix (e.g., DNA-binding dyes). When the control data are loaded into the software, the dark current correction offsets are automatically applied to each pixel in the file to correct for any inconsistencies in the detector. The spatial offsets are then automatically applied to each channel to ensure that a given pixel address in each image corresponds to the same location in the cell. The software shifts each channel of imagery with sub-pixel resolution to register it with the reference channel. An interpolation algorithm then computes the proper intensity for each pixel based on the degree of spatial shift, thus preventing artifacts such as pixel underflow as a result of the application of the compensation matrix (15).

After the control file has been loaded, dark current corrections applied and the imagery spatially corrected, the software automatically identifies the singly labeled cells, defines representative cell populations, and generates bivariate plots showing each population and its crosstalk into other channels. The software then prompts the user to confirm that the defined populations are appropriate and computes the crosstalk matrix coefficients based on the slope of the regression line within each bivariate crosstalk plot. The user can then accept the coefficients or manually make changes based, in part, on the calculated goodness of fit of the regression line.

Brightfield imagery crosstalks into other channels in a manner similar to fluorescence because of imperfect spectral filtering of the brightfield illumination and the multi-spectral decomposition filters. However, brightfield crosstalk coefficients are computed from the background around the objects rather than emission from the objects themselves. When the data file is loaded, the data analysis software automatically determines the brightfield channel from the instrument configuration parameters embedded in the data file and measures the background light level in all channels. The mean background signal from each channel is divided by the mean background signal in the brightfield channel to generate the crosstalk coefficients.

The Compensation Matrix

When all the fluorescence and brightfield coefficients have been determined, a $6 \times 6$ matrix of coefficients is
automatically compiled. Compensation matrices can be stored and applied to other data files employing the same probes, or the values in the matrix can be edited manually should control files be unavailable for whatever reason. The compensation matrix is applied to the data by solving a set of six simultaneous linear equations for each pixel in the image, based on the coefficients listed in the compensation matrix. The application of the compensation matrix is conceptually identical to standard flow cytometry, except that compensation is performed independently for each of the hundreds or thousands of pixels per cell image, rather than just once per cell.

**Application of Brightfield Gain Correction**

The final step in the compensation process involves application of the pixel gain correction values determined in step 2 from above. Pixel gain correction is applied to the brightfield channel to correct nonuniformities in brightfield illumination, helping to ensure repeatable and accurate absorbance measurements. Pixel gain correction is performed by multiplying each pixel intensity in each brightfield image by the corresponding gain correction factor determined in step 2. Absorbance measurements within each cell are made relative to the mean background illumination level through the clear sheath fluid surrounding that cell, which serves as a cell-by-cell internal control against potential variations in illumination intensity, exposure time, and other measurement factors.

**Fluorescence Cross Talk Compensation Example**

A sample of K562 cells was individually stained with probes having emission spectra centered in four different spectral channels: Ch3 (AF488), Ch4 (Cy3), Ch5 (PE-AF610), and Ch6 (DRAQ5) (Fig. 1A). Data from each sample was collected separately in the absence of brightfield illumination and then merged into a single data file serving...
as a fluorescence control file. For presentation purposes, an additional sample file was collected containing all singly labeled cells with brightfield illumination enabled (Fig. 1A). Inspection of the uncompensated image data in Figure 1A reveals that each probe generates a dominant channel image and a set of dimmer crosstalk channel images. It should be noted that the darkfield signal is typically collected only from the first 32 rows of TDI detector, which, in object space, are illuminated by the tail end of the Gaussian laser intensity distribution. The amount of fluorescence generated from this portion of the laser is extremely small and therefore does not crosstalk into the darkfield channel. Further, a 488 nm Rugate notch filter is applied over all TDI detector channels, except for the darkfield channel, during the instrument assembly process. The addition of this filter and the narrow laser bandwidth prevent any crosstalk or scatter from the laser into other channels.

Logarithmic-linear intensity plots of the uncompensated cell populations in the fluorescent channels are also shown in Figure 1A (each axis is logarithmic above 1,000 counts and linear below 1,000 counts). The slope of the best fit line defined by each population mathematically defines the crosstalk coefficient for each probe into each channel. The process of selecting singly labeled cell populations from the control file, determining the dominant channel and computing the crosstalk coefficients for all probes into all channels is automated.

In Figure 1B, logarithmic-linear intensity plots of the cell population and representative cell images after compensation are shown. Note the rectilinearity of the intensity plots and, within the compensated image gallery, the corresponding absence of “ghost” imagery outside of the dominant emission channel for each probe. Finally, representative compensated imagery from cells stained with the fluorochrome-labeled monoclonal antibodies mAb to NFkB, CD71 and CD45, and the DNA binding dye, DRAQ5, is shown in Figure 2.

### Fluorescence Intensity Measurements Using Multispectral Imagery

Once compensation has been performed, a wide variety of photometric and morphometric features can be calculated. While there are numerous applications that are enabled by the quantitative measurement of morphology, granularity, and fluorescence distribution, multispectral imagery can also be used to measure standard fluorescence intensity parameters. However, unlike standard flow cytometry object size plays a role in the sensitivity and dynamic range that can be achieved. Data will be shown that ImageStream intensity measurements have sensitivity and dynamic range comparable to or better than standard flow cytometers.

### Dynamic Range

In CCD-based systems, the size and distribution of signal within the image has a large effect on the dynamic range and sensitivity of measurements. The dynamic range of an intensity measurement is defined as the maximum signal divided by noise (16). With imagery, dynamic range is defined as the maximum signal per pixel divided by the noise per pixel, summed over the number of pixels in the image, \( N \). The summed signal increases linearly with the \( N \), but the random noise increases as the square root of the number of pixels. The general equation for the dynamic range as a function of the number of pixels, each digitized using \( M \) bits and having \( S_n \) counts of noise per pixel (\( S_n < 1 \)), is:

\[
\text{Signal}_{\text{max}} \over \text{Noise} = \frac{N \times 2^M}{\sqrt{N} \times S_n} = \sqrt{N} \times 2^M
\]

or, if the dynamic range is expressed in terms of the number effective bits of digitization and the noise level \( S_n \) is assumed to be 1 count, the equation becomes:

\[
\text{No. of Bits} = \log_2 \left( \frac{N \times 2^M}{\sqrt{N} \times 1} \right) = M + \frac{1}{2} \log_2(N)
\]

The ImageStream’s 10-bit pixels are 0.5 \( \mu \)m on a side (0.25 \( \mu \)m\(^2\) in area) in object space, meaning that a virtual point source of fluorescence from a bacterium or within a larger cell will be measured with three logs of dynamic range.
range. As the area of the fluorescent signal increases, so will the dynamic range. For example, a cell 18 μm in diameter has a corresponding image area of ~1,000 pixels. According to the equation above, the effective dynamic range of the intensity measurement of such a cell is over 30,000:1 or 15 bits.

The theoretical dynamic range equation derived above is based on assumptions about the characteristics of the signal at both the maximum and minimum of its range. To achieve full dynamic range with strong signals, the image pixels must all saturate at the same time, so the theoretical treatment assumes a uniform intensity distribution over the whole image. In most cases, this assumption does not hold. An example is a surface-labeled cell where the signal is concentrated at the perimeter of the image in the familiar “coffee ring” pattern. In such a case, saturation will be observed in the peripheral pixels before it occurs in the interior of the image and the maximum nonsaturating signal may only be one half to one third of the theoretical maximum. Nevertheless, four decades of dynamic range are routinely achieved within a given image channel.

The ImageStream system utilizes a CCD detector with 512 rows of pixels for integration. The detector was designed to allow for selectable control of the number of rows used in the integration process. Specifically, the user can select 512, 256, 128, or 32 rows for signal integration. This offers an additional decade of dynamic range between spectral channels. Further, the Gaussian intensity profile of the laser used for fluorescence excitation decreases in intensity by more than a decade for the lower stage selection levels, adding another decade of dynamic range between channels on the same detector. By using only 32 stages in the 488 nm spectral channel, in conjunction with spectral filtration, saturation of the darkfield imagery can be avoided despite the fact that the scattered laser light forming the darkfield imagery may be more than 100,000 times brighter than the fluorescence being emitted from the same cell and imaged in a different channel.

**Signal Resolution**

The fluorescence detection limit is a measure of the minimum number of molecules that must be present in or on an object to be detected and resolved from system noise. Since signal levels are very low, the detection process involves the use of statistical criteria to resolve a distribution of signals from a distribution of noise. An analogous situation exists for the resolution of two populations having similar intensities.

In classical optical design, the Rayleigh criterion states that a point of light is said to be resolvable from a neighboring point of light if its peak intensity (central maxima) point falls within the first minima of its neighbor (17). When viewing both points together, this results in a slight dip in the combined intensity profile of the two points. This same concept can be employed to the measurement of normal distributions of signals resulting from the measurement of nonfluorescent and weakly fluorescent particles.

When viewing distributions of intensity measurements on a histogram plot, one can infer that two populations are present if there is a dip present in the plot (Fig. 3). In place of the central maxima—first minima criterion, one can substitute a proposed distribution resolution metric, \( R_d \), to infer the existence of two populations. The distribution resolution metric, \( R_d \), is defined as the difference in

**Fig. 3.** Distributions for different levels of the resolution metric \( R_d \). Four simulated distributions (thick line) resulting from the measurement of intensities for two simulated underlying populations are shown. Each plot shows the underlying populations (thin lines) and the \( R_d \) value if the populations were measured separately and overlaid. At \( R_d \) values of 0.5 and 1.0, it is not possible to determine if the resulting distribution is a function of two underlying species. At an \( R_d \) value of 1.2, the resultant distribution begins to show a dip, indicating that two species may be present. An \( R_d \) value of 1.5 may be considered a distribution resolution limit to ensure two separate species are present in the measurement.
the means $M_d$ and $M_u$ of two populations (unlabeled and dimly-labeled, respectively), divided by the sum of the standard deviations of the distributions, $\sigma_u$ and $\sigma_d$, as shown below:

$$R_d = \frac{M_d - M_u}{\sigma_d + \sigma_u}$$  \hspace{1cm} (3)

Variants of Eq. (3) have been used in other applications, including the analysis of gene expression microarray data to select genes whose expression level best discriminates between two classes of samples (18). The expression incorporates measurement noise and is independent of the type of detector being used or its relative gain settings. The degree of separation between overlapping populations as $R_d$ increases is illustrated in Figure 3. Four simulated distributions are shown (thick lines) each of which represents a large number of intensity measurements taken from a population of objects. Each distribution contains contributions from two underlying sub-populations (thin lines) that overlap to varying degrees. A progression is shown in which the mean intensities of the sub-populations are increasingly separated, as indicated by the increasing $R_d$ values. At $R_d$ values of 0.5 and 1.0, it is not possible to determine if the resulting distribution is a function of two underlying species (e.g. nonfluorescent and dimly fluorescent beads, thicker black and grey lines) or just a larger population of a single species (e.g. nonfluorescent beads). At an $R_d$ value of 1.2, the resultant distribution begins to show a dip, indicating that two species may be present (close to the Rayleigh Criterion). With an $R_d$ value of 1.5 (lower right-hand plot), the resulting distribution shows a marked dip and one may conclude that two distinct sub-populations are present.

**Effects of Pixel Count and Multimode Imagery on Detection Limit**

Equation (3) can be modified to provide an expression for the minimum signal, in counts, that can be detected by an imaging system. The numerator of Eq. (3) is the difference between the mean signals collected from dim fluorescent particles, $M_d$, and nonfluorescent particles, $M_u$. When corrected for dark current, the unlabeled mean, $M_u$, goes to “0”, leaving only $M_d$ in the numerator. The denominator of Eq. (3) is the sum of the standard deviations of the measurements of dim and nonfluorescent particles. Since the standard deviation of a dim population of fluorescent particles is dominated by system noise, it is nearly identical to the standard deviation obtained by measuring the nonfluorescent particles. Therefore, at the detection limit, the denominator can be approximated as $2\sigma_u$, or two times the system noise. As described in the derivation of Eq. (1), the measurement noise, $\sigma_u$, is the product of the individual pixel noise $S_n$, and square root of the number of pixels $N$, involved in the measurement. Rearranging the terms and using a value of 1.5 for $R_d$ yields Eq. (4), which provides $M_d$, the minimum detectable signal in counts.

$$\frac{M_d - M_u}{\sigma_d + \sigma_u} = R_d \Rightarrow \frac{M_d - 0}{2\sigma_u} = R_d \Rightarrow M_d = R_d2\sigma_u$$

rearranging terms and combining with Eq. (1) yields:

$$M_d = 3 \times \sqrt{N} \times S_n$$  \hspace{1cm} (4)

Equation (4) provides a simple relationship between the minimum detectable signal in counts, the number of pixels used in the measurement, and the noise per pixel. For a theoretical prediction of photonic sensitivity, the equation for $M_d$ must be converted from counts to molecules of fluorochrome. This requires the selection and characterization of a specific fluorochrome, including excited state lifetimes, absorbance cross sections, bleaching probabilities, excitation and emission spectra, etc. Likewise, an expression for theoretical sensitivity must include a complete characterization of the instrument, including the collection system numerical aperture and field size, optical filtering efficiencies, excitation illumination power and intensity profiles, sample transport integration times, etc. Theoretical sensitivity predictions are best handled by Monte Carlo simulations due to the large numbers of probabilistic factors involved and are beyond the scope of this study.

Application of Eq. (4) provides some insights into the detection capabilities of image-based cell analysis instruments. For example, in the case of an object covering 5 pixels, the minimum signal that can be measured is ~5 counts (using an actual measured per pixel noise of 0.7 counts), whereas an object with an area of 500 pixels requires a minimum detectable signal of 47 counts, or ten times larger. The implication is that for imaging systems, unlike conventional PMT-based flow cytometers, the sensitivity is less for the detection of larger objects. Conversely, a signal from a small object like a bacterium that is below the detection limit of a flow cytometer may be detectable in an imaging system because its signal is concentrated in just a few pixels, thereby lowering the effective measurement noise.

Absent from the treatment above is the effect of quantization noise, which can have a profound effect on the detection limit (19). To illustrate why this is so, consider the 500 pixel object discussed above. At the theoretical detection limit, the average signal per pixel would be as low as 0.1 counts (47 counts/500 pixels). The presence of the fractional count of signal per pixel, combined with the pixel noise, will increase the statistical likelihood that any given pixel value will rise above 1 count. However, most signal segmentation algorithms will fail to detect such a small signal or, if they are tuned to do so, they will misclassify background noise (both patterned and random) as signal.

The ImageStream system provides robust object segmentation while achieving the theoretical detection limit despite quantization noise by taking advantage of the.
The mask used to define the signal area may be simple or complex depending upon the nature of the objects being imaged. For example, if an experiment requires the quantitation of only the cytoplasmic component of a fluorescent probe, the ImageStream system can be directed to use a Boolean combination of the brightfield and nuclear segmentation masks to define a ring-like search area encompassing only cytoplasm (21). Using biological context and existing information from other channels to narrowly define the search space has the benefit of substantially reducing the number of pixels in the search area for a given object. This, in turn, can reduce the noise of the measurement, allowing the ImageStream to detect very dim signals. In a similar manner, the use of selective masking allows the exclusion of undesired signal components, e.g., background staining due to nonspecific binding in FISH-probed cells (22). Using these techniques, the ImageStream can match or surpass the sensitivity of a conventional PMT-based flow cytometer.

**Comparison of Fluorescence Sensitivity**

The preceding discussion provides a theoretical basis for factors affecting sensitivity and provides some insights into how alternate modes of imagery can be used to improve the detection of very low signals. It also provides the basis for comparing the sensitivities of different instruments through the use of the signal resolution metric, $R_d$. $R_d$ is dimensionally unitless and can be used to compare the photonic sensitivities of different instrumentation platforms, different instruments of the same type, or to measure changes in photonic sensitivity over different operating conditions for a given instrument (23,24). It incorporates noise sources, it is easily measured and provides a single number for comparison. $R_d$ can be measured by running commonly-available calibration bead sets of different brightness levels (Fig. 5). Ideally, the bead set includes an unlabeled population and at least one dimly fluorescing mate to the size of labeled organelles or yeast. With the 6 l parameter bead size was chosen to approximate the diameter of a cell nucleus for one comparison. In another comparison, a 3 l diameter bead size was chosen to approximate the size of labeled organelles or yeast. With the 6 l beads, the $R_d$ values between the two dimmest peaks in the FL3, FL4, and FL5 spectral bands averaged 1.5 times greater in the ImageStream data relative to the flow cytometric data (Fig. 5A). With the smaller 3 l diameter beads, the $R_d$ values in the ImageStream data averaged three times higher than the conventional flow cytometer (Fig. 5B). It should be noted that for the purposes of this
study, the ImageStream system was configured for the maximum resolution of dim peaks, rather than maximum linear dynamic range. As described by Eq. (2), the dynamic range of the system can exceed 15 bits. However, deviations from linearity can occur when individual pixels within the brightest bead images are allowed to saturate due to high laser power and/or detector gain settings. The effects of fractional pixel saturation are evident in the spacing and shape of the brightest peaks of the 6 μm and particularly the 3 μm ImageStream bead data.

To compare the sensitivity of the ImageStream to itself as a function of bead size, a normalization process is required because the fluorochrome count on the 3 and 6 μm beads is not matched. The flow cytometer's sensitivity is assumed to be largely independent of object size for the 6 and 3 μm beads, so the normalization factor is simply the ratio of the $R_d$ values of the two different bead sizes as measured on the flow cytometer. When this factor of 2.38 is applied to the ImageStream data to normalize for the differing amounts of fluorochromes on

![Flow Cytometer vs ImageStream Comparison](image)

**Fig. 5.** Comparative sensitivities of the ImageStream and an 18-bit flow cytometer. Higher sensitivity is indicated by higher $R_d$ values (shown on each plot). A: Rainbow calibration particles of 6 μm diameter were divided into two tubes and run on a digital 18-bit FACS analyzer (column A) and the ImageStream (column B). The detectors used to read FITC (in green), PE (in orange), and PE-Cy5 (in red) fluorescence were compared for their ability to distinguish two dimly fluorescing bead populations by calculating the separation between the populations and indicating that value in the histogram. B: The same analysis was done with 3 μm rainbow beads. For the data generated on the ImageStream, the brightest three sets of beads have events that saturate the camera pixels; however, this does not affect the separation metric.
the larger and smaller diameter bead sets, the \( R_d \) value drops from 31.2 to 13.1 for measurements of the 3 \( \mu \)m bead set (in the FITC channel). This is twice the \( R_d \) value obtained from the 6 \( \mu \)m beads. As predicted by Eq. (4), the ImageStream's sensitivity is inversely proportional to the diameter of the test beads, since the number of pixels in the bead image scaled as the square of the diameter.

Using the \( R_d \) values from the ImageStream measurements of 3 and 6 \( \mu \)m diameter bead intensities, it can be shown that the conventional flow cytometer is invariant to object size. If one divides the ImageStream's measured \( R_d \) ratio of 4.8 (3 to 6 \( \mu \)m beads in FITC channel, \( 31.2/6.5 = 4.8 \)) by the bead size factor of two (6 \( \mu \)m/3 \( \mu \)m = 2), a correction factor of 2.4 is applied to the flow cytometric \( R_d \) measurements. When this is done, the \( R_d \) value of 10.4 measured on the conventional flow cytometer for the 3 \( \mu \)m beads is normalized to 4.3 (10.4/2.4 = 4.3), which is very close to the 4.4 \( R_d \) value as measured on the 6 \( \mu \)m beads.

**DISCUSSION**

The process of spectral compensation in multispectral imaging is complicated by the added dimensions of space,
multiple image collection modes, and correction of detector anomalies at the pixel level. In the ImageStream system, these factors are addressed through a series of automated calibrations that are largely invisible to the user. Like conventional flow cytometry, the quantitation of spectral overlap relies upon the aggregate signal collected from a group of objects and their distribution of light into each channel. However, the application of spectral correction is done on an individual pixel level as opposed to the object level. During the compensation process, each channel is brought into sub-pixel spatial registration prior to application of the correction matrix. This step ensures that spectral corrections are properly applied at each location in the imagery. In this manner, spectral overlap from FISH spots, sub-cellular structures, marker capping, or any other sub-cellular feature is accurately represented after compensation, regardless of the imaging mode, thereby preserving the underlying morphological and photometric information. After compensation, the imagery is analyzed with the IDEAS software package where over two hundred photometric and morphological features are automatically calculated for each cell for the determination and identification of biologically relevant events or populations. Amongst possible examples are quantitation of nuclear translocation, image-based determination of stages of apoptosis, organelle identification, and colocalization of signals (25,26).

The ImageStream system demonstrates fluorescence sensitivity equal to or better than conventional flow cytometers. This is in part due to the longer signal integration times afforded by the TDI collection process, which increases signal strength without adding significant noise. As discussed theoretically, and demonstrated empirically, the sensitivity is also a function of object size. In general, for a fixed number of fluorescently labeled molecules, the sensitivity is higher for smaller objects or features where the molecules are concentrated. The sensitivity benefits of TDI are complemented by the simultaneous collection of multiple imaging modes. As shown, the spatial information contained in the alternative multispectral images (brightfield, darkfield, nuclear stain, etc.) can be used to direct the search for weak signals and remove background noise in a fluorescence channel that could not otherwise be isolated and quantitated. The ImageStream system provides a high level of sensitivity, a wealth of information derived from multiple imaging modalities and the throughput to apply statistical rigor to answer new questions in cell analysis.

LITERATURE CITED